USE OF MICROBIAL ANTAGONISTS TO CONTROL POSTHARVEST BLACK ROT OF PINEAPPLE FRUIT

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ABSTRACT

The microbial population on the pineapple fruit shell, black rot (*Chalara paradoxa* (De Seyn.) Sacc. = *Thielaviopsis paradoxa* (De Seyn.) Hohn., teleomorph: *Ceratocystis paradoxa)* incidence and severity were monitored during a 14-month period. There was low variability in microbial counts from month to month, except in November when rainfall was high. Yeasts made up the majority of the microbial population and the rest was filamentous fungi. Black rot incidence in wet fruit was negatively correlated with filamentous fungi count in wet fruit, suggesting that some of the filamentous fungi on the fruit were washed away, predisposing the fruit to black rot. Rainfall did not play a major role in black rot incidence and severity. Total microbial counts were correlated to rainfall in the month of harvest. A naturally occurring, epiphytic antagonist population is present on the pineapple fruit as evidenced by the reduction of black rot severity in fruit treated with pineapple fruit wash water and black rot spores. The most frequently isolated yeasts from the pineapple fruit shell were able to inhibit *C. paradoxa* growth *in vitro*. The most promising yeast isolate was Pichia (*Pichia guilliermondii*). A yeast mixture containing all five yeast isolates individually tested was able to reduce black rot severity by half compared to the control. The use of Pichia or the yeast mixture was compatible with current industry practice of holding fruit at a low temperature (10°C) and the use of Bayleton. Combining the isolate Pichia or the yeast mixture with a half dose of Bayleton resulted in complete control of black rot comparable to control achieved with a commercial dose of Bayleton. The yeast isolate Pichia and the yeast mixture containing all five isolates tested were able to reduce spore germination, germ tube length, and dry matter weight of *C. paradoxa*. The mode of action by Pichia appeared

to be competition for space and nutrients. As for the yeast mixture, mode of action appeared to be competition for space.

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LITERATURE REVIEW

1.1 Introduction

Pineapple is still a major fruit crop in Hawaii despite the recent decline in production. In 1997, production was 324,000 tons, valued at $91.7 million and pineapple still makes up almost 90 percent of the total fruit production in the Hawaiian islands (Statistics of Hawaiian Agriculture, 1999).

The pineapple is prized both as fresh or canned fruit. Most of the production in Hawaii is as canned fruit (221,000 tons) but fresh fruit (103,000 tons) commands a higher price. The farm price of fresh fruit is $618 per ton compared to $127 per ton canned (Statistics of Hawaiian Agriculture, 1999). According to Maui Pineapple Co., the retail price of whole fresh fruit is $2.99 per fruit on the West Coast and $3.99 per fruit on the East Coast compared to a canned fruit price of $0.89 per 20 oz can (US International Trade Commission, 1995).

A postharvest fresh pineapple disease problem is black rot (Figure 1.1), caused by the fungus *Chalara paradoxa* (De Seyn.) Sacc. = *Thielaviopsis paradoxa* (De Seyn.) Hohn., teleomorph: *Ceratocystis paradoxa* (Rohrbach and Apt, 1986). The disease starts in the field as the causal pathogen enters the fruit through the cut peduncle, other wounds or natural openings in the fruit shell. Disease development can be delayed by keeping the fruit in cold storage, but once the fruit is taken out of storage and is put on the display shelves, the disease progresses rapidly. Currently, fruit are treated with Bayleton (Bayer Corp., Michigan, MO). However, since fungicides registered for postharvest use are being restricted (Wilson and

Wisniewski, 1989; Droby et al., 1991; Ragsdale and Sisler, 1994; Biles, 1995; El-Ghaouth and Wilson, 1995; Aked, 1997; Taylor, 1997; Koomen, 1997), even Bayleton will not be available for use in the long term.

1.2 Black Rot of Pineapple Fruit

There are a number of diseases that become a problem in the fruit postharvest. Infection of the fruit by the disease-causing microorganisms may take place anytime during growth of the fruit in the field up to the time it is brought into the packinghouse for grading and packing. Some of the diseases are cosmetic such as *Penicillium* sp. mold on the cut fruit peduncle. Black rot results in heavy losses in fruits sent to the fresh fruit market or otherwise held in boxes or bins for some days before processing (Larsen, 1910; Roldan, 1925; Dickson et al., 1931; Liu and Rodriguez-Marcano, 1973; Rohrbach and Apt, 1986).

1.2.1 Causal organism

This disease is caused by *Chalara paradoxa* (De Seyn.) Sacc. = *Thielaviopsis paradoxa* (De Seyn.) Hohn., teleomorph: *Ceratocystis paradoxa*.

1.2.2 Occurrence

All the principal pineapple growing countries have reported the disease(Roldan, 1925; McKnight, 1941; Liu and Rodriguez-Marcano, 1973; Snowdon, 1990). Frequently the reports relate to fruit picked green and shipped long distances to market.

Aside from attacking the fruit, the fungus also causes rotting of plant material in the field under conditions of high moisture and humidity (Larsen, 1910; Roldan 1925). The fungus has also been reported to be parasitic on sugarcane (Smith, 1904; Roldan, 1925;

Cook, 1933), banana (Larsen, 1910; Stover, 1972), cocoa (Dade, 1928) and various palms including coconut and oil palm (Ocfemia, 1924; Roldan, 1925).

Observations made by Dole Hawaii indicate black rot on fresh fruit occurs sporadically throughout the year especially when fruit referred to as "leakers" are more prevalent. "Leakers" are those fruits that secrete lot of fluid at the broken peduncle end (R. McCormack, personal communication, 1992; Paull and Reyes, 1996). More "leakers" are seen when rain is more prevalent and overcast or with cool nights, warm and overcast days. Furthermore, the disease is observed more on washed than unwashed fruit. It has commonly been observed that washed agriculture produce develops more rot than unwashed produce (Chalutz and Wilson, 1990). When fruits and vegetables are washed, there is a possibility that a microbial population that imparts resistance to rotting is removed. Washing probably also affects rot resistance in other ways, such as removing protective waxes.

1.2.3 Symptoms

Several authors have described "black rot" of pineapple fruit (Larsen, 1910; Ocfemia, 1924; Roldan, 1925; Linford and Spiegelberg, 1933). The fruit tissue when affected takes on a water-soaked appearance, becomes a darker yellow than normal tissue (Figure 1.2) and has a characteristic odor resembling ethyl acetate. The genus *Chalara* is well known to produce fruity smelling volatiles during fruit rotting (Collins and Morgan, 1962; Collins and Kalnis, 1965). The fruit is very soft and juicy, even in the early stages of decay, and the flesh becomes so thoroughly disintegrated that it yields to the slightest pressure. The softening of the fruit is thought to be due to cellulolytic and proteolytic enzymes detected in infected fruit exudates (Adisa, 1987). Healthy fruit exudates have traces of cellulase activity, therefore the

high production of cellulases in exudates from infected fruit could be stimulated as a result of the presence of cellulosic cell constituents, thereby inducing *C. paradoxa* to be cellulolytic. Other cell wall degrading enzymes in the exudate may also be involved.

A distinguishing feature of the rot is black spore formation that takes place over the surface of diseased tissue when exposed to air (Figure 1.3). This black formation consists of the dark colored macrospores. The macrospores develop so freely that the entire exposed surface becomes black. In advanced stages of rot, macrospores may occur within the fruit, especially along the core. In most cases it is necessary to cut open the fruit and expose the affected tissue to atmospheric conditions for about 24 hours before the black spores become apparent. In the final stages of rotting, when the fruit is disintegrating, the whole fruit becomes covered with spores. The rot is often accompanied by fermentation due largely to organisms other than *Chalara* that gain entrance or are already present in tissue. In advanced stages, the fruit, as a rule, become infested with a swarm of vinegar fly maggots (*Drosophila ampelophila*) and fruit beetles (*Carpophilus humeralis*) (Larsen, 1910)..

1.2.4 Biology

C. paradoxa may be classified as a facultative parasite (Larsen, 1910). This implies that it has the general qualities of a saprophytic organism, but under certain conditions, can behave as a parasite. Its saprophytic nature is apparent as it is found in soil (Contois, 1952; Rashid, 1975), in dead roots and leaf trash, or in ripe fruit such as pineapple, banana and mango (Roldan, 1925; Liu and Rodriguez-Marcano, 1973; Snowdon, 1990). The fungus' parasitic character appear once it gains entrance to healthy tissue (e.g. pineapple leaf, sugarcane stalk) where it rapidly destroys the plant tissue. The fungus seizes any opportunity

to invade wounded tissues. The fungus is generally considered a wound parasite and may attack the green or ripe pineapple fruit after harvest. It may gain entrance through growth cracks, the cut peduncle and injury caused by insects and rats or mechanical bruising (Smith, 1904; Cobb, 1906; Larsen, 1910; Roldan, 1925; Liu and Cortes-Monllor, 1972; Chang and Jensen, 1974; Rohrbach and Apt, 1986).

Fruit can experience hard impacts at certain points along the packing line and these may cause some type of bruise to the fruit, predisposing it to pathogen attack (Timm and Brown, 1991). However, the fungus typically enters the core through the broken peduncle of the fruit and advances upwards as a cone, progressing along the core more rapidly than through the flesh. The fruit may finally be reduced to an empty shell containing little but the blackened fibers of the vascular bundles, all the juices having drained away. This very soft rot suggested the name 'water blister', used in Queensland (Linford and Spiegelberg, 1933). The fungus can also force its way into the fruit with or without injuries provided moist atmospheric conditions prevail (Larsen, 1910; Roldan, 1925).

1.2.5 Control

Although black rot is not a problem with commercially processed fruit (harvested fruit processed within 24 to 48 hours), infection is a major problem when fruit are held at ambient temperatures for longer than three days. Refrigeration at 8°C retards, but does not prevent, infection and development of *C. paradoxa*. Thus, fruit held at ambient temperatures at the retail market after refrigeration for 2 weeks or longer during shipment can still show severe rot problems (Rohrbach and Apt, 1986).

Inoculum levels of *C. paradoxa* on harvested fruit vary considerably, with 0 to 100% of the fruit becoming infected (Rohrbach and Apt, 1986). Without a method to predict inoculum levels, all commercial fresh-market fruit must be treated.

In the past, control was achieved by careful packing of unbruised fruit and leaving as long a peduncle as possible on the fruit. This practice is based on the premise that the fungus gains entrance usually through the broken peduncle (Larsen, 1910; Roldan, 1925). Sun drying of the cut end of the fruit stalk appears to be beneficial. In Australia, Dickson and Simmonds (1932) secured good control of the disease by applying salicylic acid or benzoic acid to the cut end of the stem within five hours after cutting the peduncle. For commercial application, an alcoholic solution of benzoic acid of not less than 10% strength, or a mixture of one part acid with not more than four parts of kaolin is recommended (Dickson et al., 1931). Borax, boric acid or salicylic acid also give good control but are so far not approved for postharvest use in Australia(Dickson et al., 1931).

In Hawaii, control of black rot was achieved with Benlate (1,200-2,400 ppm a.i.) and fruit were dipped or sprayed before packing (Cho et al., 1977). Fruit must be treated within 6 to 12 hours of harvest to prevent infection. Postharvest use of Benlate on pineapple was discontinued following voluntary withdrawal by DuPont. Bayleton is the current chemical used for fruit and crown dips to control black rot in pineapple. Other chemicals being tested for postharvest use include Elite, Fungalor, Mertect and Nustar (G. Taniguchi, personal communication). Except for Elite, the tested chemicals are not as effective as Bayleton in controlling black rot. Elite is superior to Bayleton in terms of controlling the disease, however it causes phytotoxic symptoms (G. Taniguchi, personal communication).

1.3 Alternative Control Measures

Public pressure to limit the use of synthetic fungicides for the control of postharvest diseases of fruits and vegetables is increasing (Droby et al., 1991; Ragsdale and Sisler, 1994; Biles, 1995; El-Ghaouth and Wilson, 1995; Aked, 1997). Concerns regarding chemical use include: 1) the increased development of fungicide-resistant strains of phytopathogens, 2) limited time during which chemicals can be used in the period between harvest and consumption, 3) problems of access of chemicals to quiescent infections (Dodd et al., 1989), and, 4) increased health and environmental concerns. There is therefore a need for safe and effective alternative methods to control postharvest diseases and imperative to explore alternative control methods. One approach is biological control using microbial antagonists and microbial antagonist-low dose fungicide combinations (Droby et al., 1991, 1993; Chand-Goyal and Spotts, 1996, 1997). The availability of microbial antagonists to control postharvest rots would mean less chemicals need be used, therefore enhancing food safety and reducing potential environmental damage.

Biological control has been defined as a reduction in the pathogen inoculum or its disease-producing capacity by action of one or more organisms other than humans (Cook and Baker, 1983). The use of antagonistic microorganisms have proven to be effective against a variety of postharvest diseases of fruits and vegetables (Janisiewicz, 1988a,b, 1991; Janisiewicz and Roitman, 1988; Droby et al., 1989, 1991; Wilson and Chalutz, 1989; Wilson and Wisniewski, 1989; Jeffries and Jeger, 1990; Huang et al., 1992; Janisiewicz and Marchi, 1992; Torres, 1992; Wisniewski and Wilson, 1992).

The postharvest environment offers a unique opportunity for biological control agents to function. There are three factors that indicate that biocontrol strategies in the postharvest area may be exceptionally productive (Wilson and Pusey, 1985; Jeffries and Jeger, 1990): 1) exact storage conditions can be established and maintained to suit the operation of a biocontrol agent; 2) the biomass of the harvested produce is less than that of the standing crop, thus only a limited surface area needs to be treated; and 3) there is a high net cost of harvested produce, justifying expenditure on control. If biological control methods cannot be successfully applied in the postharvest environment, there is less hope of achieving this in other environments (Jeffries and Jeger, 1990) .

Biological control of postharvest diseases has developed as a realistic strategy to control a number of postharvest diseases in fruits. One of the first reports is the control brown rot of stone fruit caused by *Bacillus subtilis* (Pusey and Wilson, 1984). Later, *Pseudomonas cepacia* (Janisiewicz and Roitman, 1988) and a saprophytic strain of *Pseudomonas syringae* (Janisiewicz and Marchi, 1992) can control gray mold and blue mold in apples and pear. Mold on citrus fruit can be controlled by *Bacillus pumilum* (Liang and Liu, 1989; Huang et al., 1992). Brown spot of pear is controlled by *Pseudomonas fluorescens* (Montesinos et al., 1996). Unidentified bacterial isolates significantly reduce black rot, heart/root rot, and fruitlet core rot of pineapple fruit (Torres, 1993). Bacteria are not the only microorganisms reported to control postharvest fruit rots, antagonistic yeast isolates including *Pichia guilliermondii* (formerly known as *Debaryomyces hansenii*) and *Aureobasidium pullulans* control green mold, blue mold and sour rot of citrus (Droby et al., 1989; Wilson and Chalutz, 1989; Chalutz and Wilson, 1990) and *Cryptococcus laurentii* for

the control of gray mold on apple (Roberts, 1990). Blue mold in apple is successfully controlled with a mixture of yeast antagonists (Janisiewicz, 1996). Several mixtures of yeasts are superior to an individual antagonist. There are also reports on biocontrol by other fungi. Lim and Rohrbach (1980) reported that interfruitlet corking, leathery pocket, and fruitlet core rot in pineapple were significantly reduced by red-pigmented strains of *Penicillium funiculosum*.

More of recent work have shown that some of the most promising antagonists can be used in conjunction with low dose fungicides or can be integrated with cultural methods in order to control fruit decay. Droby and his co-workers (1998) was able to show that Aspire (a biological control product containing the yeast *Candida oleophila* as the active ingredient) combined with 200 $\mu g\ ml^{-1}$ thiabendazole (TBZ) often reduced the incidence of decay caused by green and blue molds in citrus, as well as a conventional fungicide treatment. Chand-Goyal and Spotts (1996) reported that *Cryptococcus laurentii* HRA5 and *Rhodotorula glutinis* HRB6 combined with a low dose of TBZ gave significantly better disease control than either TBZ alone or yeast alone and was comparable to disease control achieved using a commercially-recommended high dose of TBZ. Sugar and his co-workers (1994) reported that early harvest, fruit having low N and high Ca, yeast or yeast plus fungicide treatments and controlled atmosphere storage reduce the severity of blue mold and side rot in pears.

Physical treatments can enhance the biological control activity of an antagonist. Huang and his co-workers (1995) found that heat treatments enhance the biocontrol activity of a strain of *Pseudomonas glathei* against green mold on oranges. Physical methods have been used (e.g. cold and heat treatments), however, the tested methods are not always

applicable to a number of fruits and vegetables. Oftentimes, the developed procedure is injurious to some commodities (Couey, 1989; Paull, 1990; Barkai-Golan and Phillips, 1991; Klein and Lurie, 1992; Paull and McDonald, 1994).

Various treatments applied to fruits and vegetables after harvest and during processing affect the fruits' epiphytic microbial populations significantly (Chalutz and Wilson, 1990; Droby et al., 1991). It has been observed that pineapple black rot occurs more commonly on washed rather than unwashed fruit. It is possible that certain beneficial organisms have been removed. These organisms could therefore be investigated as to their potential as biocontrol agents against postharvest diseases.

1.4 Biological Control Approaches

A few antagonists that have been shown to control plant pathogens have been successfully transferred from the laboratory to the field or used postharvest Two primary barriers have prevented commercialization: 1) antagonists are less effective than chemical control procedures, and, 2) a lack of economic incentives (Wilson and Wisniewski, 1989). Upon selection of an antagonist, a considerable investment of effort, time and money is required to establish whether it has commercial potential and can be registered as a proprietary product. Therefore it follows that careful deliberation must be given to the isolation, screening, and selection process for potential antagonists.

The following is a list of desirable characteristics of a biological control agent for postharvest diseases (Wilson and Wisniewski, 1989): 1) genetically stabile, 2) effective at low concentrations, 3) not fastidious in its nutrient requirements, 4) able to survive well under adverse environmental conditions (including storage environments), 5) efficacious against a

wide range of pathogens on a variety of fruit and vegetables, 6) amenable to growth on an inexpensive medium in fermenters, 7) preparable in a form that can be effectively stored and dispensed, 8) non-productive of secondary metabolites that may be deleterious to humans, 9) resistant to pesticides, 10) compatible with other chemical and physical treatments of the commodity, and 11) non-pathogenic against the host.

Among potential antagonists, the yeasts deserve special attention since yeasts can (Janisiewicz, 1988a): 1) colonize the surface for long periods of time under dry conditions, 2) they produce extracellular polysaccharides that enhance their survivability and may restrict colonization sites and the flow of germination cues to fungal propagules, 3) they rapidly use available nutrients and proliferate, and 4) are minimally impacted by pesticides.

1.4.1 Isolation

There is no relationship between the origin of an antagonist and its effectiveness against foliar pathogens (Spurr, 1981). A similar situation appears to exist with antagonists effective against postharvest pathogens. So far, effective antagonists have been found in the soil (Pusey and Wilson, 1984), on fruit and other plant surfaces (Kerr, 1980; Wilson et al., 1993;). However, if commercialization is a goal, then it could be argued that it would be more acceptable to consumers if the antagonists were selected from the environment where they will be applied.

1.4.2 Screening-primary and secondary

Screening of potential antagonists may be done *in vitro* or *in vivo*. *In vitro* screening of microorganisms biases the selection process toward those antagonists that produce antibiotics (Wilson et al., 1993). Since antibiotic-producing antagonists may not be

acceptable as "food additives" when used on commodities to control postharvest diseases, *in vivo* screening is advisable. This would allow the recognition of other modes of action besides antibiosis (Wilson et al., 1993) and alleviate the problem presented by a poor correlation between *in vitro* activity and *in vivo* performance.

1.5 Mechanisms of Biological Control

It is important to understand the mechanism by which a biological control agent works. Firstly an understanding will allow the development of more reliable procedures for the effective application of known antagonists, and secondly, it should provide a rationale for selecting more effective antagonists (Wilson and Wisniewski, 1989). In the postharvest arena, antibiotic production appears to be a major mechanism of action of many of the antagonists identified so far (Chalutz et al., 1988 as cited by Wilson and Wisniewski, 1989). This is inherent in the *in vitro* method that most investigators have used to screen isolates. However, in recent years, there has been a shift from the use of antibiotic-producing bacteria toward the development of non-antibiotic-producing antagonistic yeasts for the biological control of postharvest diseases (Wisniewski and Wilson, 1992). More and more yeast antagonists, with a different mechanism of action, are being evaluated (Droby et al., 1989; Chalutz and Wilson, 1990; Janisiewicz, 1996; Piano et al., 1997). This is due in part to the selection strategy developed by Wilson and his co-workers (1993), wherein fruit wounds were utilized to screen for potential antagonists to postharvest rot organisms from unidentified microbial populations on fruit surfaces.

Many of the evolving concepts for mechanisms of action for biological control of plant diseases in general will eventually apply to biological control in the postharvest arena. These

mechanisms include: colonization, competition, antibiosis, mycoparasitism, fungistasis (Campbell, 1989).

1.5.1 Colonization and inoculum

Colonization of a plant can only occur from inoculum either resident in the environment or brought there by wind, water, animals or man. Rapid growth of an inoculum in an infection court will prevent the growth of the pathogen. An example is the control of *Botrytis cinerea* by *Candida saitoana* in apple fruit (El-Ghaouth et al., 1998).

1.5.2 Competition

Competition occurs when two (or more) organisms require the same thing and the use of this by one reduces the amount available to the other (Campbell,1989). Thus microorganisms may compete for nutrients, oxygen, space, or light (in the case of autotrophs). Nutrient competition is seen when one microorganism (because of better uptake mechanisms or better extracellular enzymes) gets most of the nutrients and grows, while the other has insufficient and dies. This is known for both carbon and nitrogen sources. An important point of the definition of competition is the deprivation of one of the organisms: if there are excess nutrients so that all have enough, then there is no competition. Therefore, microorganisms cannot compete for water. They may need water but they do not really affect the amount available to them or the distribution, as a higher plant would do. Microbes may however compete for space in which water levels are suitable or optimum. Examples include yeasts antagonists to control postharvest fruit rot (Droby et al., 1989; Chalutz and Wilson, 1990; Janisiewicz, 1996; Piano et al., 1997).

Competition for oxygen is a possible mechanism of action that is seen in some germinating seeds. Cereal seeds sown into high organic matter soils or where straw is decomposing are short of oxygen and this makes them liable to leak nutrients, usually from the micropyle. This region is then colonized by a variety of organisms but especially the fungus *Gliocladium* which grows on the exudates and increases oxygen deficiency. Oxygen competition may also account for some of the disease control obtained from an increase in general microbial activity when the soil is ammeded with organic matter.

A particular form of nutrient competition has been proposed as another mechanism of biological control (Swinburne, 1986). This involves competition for ferric iron. Many organisms produce special iron-chelating compounds called siderophores in iron-limited environments, such as arable soils which are on limestone rocks or which are limed to improve the aggregate structure in clay soils. These soils have high pH that can precipitate most of the ferric iron as hydroxide. Siderophores are produced by microorganisms to assist in the uptake of iron. Different siderophores differ in their affinity for iron so there can be competition among siderophores and those with the highest affinity will sequester all or most of the iron. Therefore, if an antagonist has a better siderophore than the pathogen, then the latter will be deprived of iron and will grow less well.

1.5.3 Antibiosis and endolysis

This mechanism of control involves enzyme production by the antagonist microorganism. The enzyme causes lysis and the complete or partial destruction of the cell. This has been seen in the control of postharvest rots with bacteria (Pusey and Wilson, 1984;

Janisiewicz and Roitman, 1988; Liang and Liu, 1989; Huang et al., 1992; Janisiewicz and Marchi, 1992 ; Montesinos et al., 1996).

According to Campbell (1989), lysis may be one of two types. The first type is endolysis, also called autolysis and could include programmed cell death. Endolysis is the breakdown of the cytoplasm of the cell by the cell's own enzymes following death. This breakdown may be caused by nutrient starvation or by antibiotics or by toxins. This does not usually involve the destruction of the cell wall. Programmed cell death, also known as apoptosis, is the process of physiological cell death (Fesus et al., 1991). Also known as cell suicide, apoptosis, is a response to environmental information (Williams, 1991). A second type is exolysis, also called heterolysis, and involves the destruction of a cell by the enzymes of another organism. Typically, exolysis is the destruction of the walls of an organism by chitinases, cellulases, etc. and this frequently results in the death of the attacked cell.

The observable effects of antibiotics in culture are as varied as their origins and their chemical nature. These effects include: reduction or cessation of growth or sporulation, reduction in germination, distortions of the hyphae of the affected fungus, changes in branching patterns of colonies, production of specialized growth forms, deposition of assorted by-products from the affected metabolism. Effects of antibiotics may occur at some distance from the organism producing them.

1.5.4 Mycoparasitism and exolysis

Antagonists may simply operate by using the pathogen as a food source (Campbell, 1989). If the pathogen is a fungus, then the antagonist is called a mycoparasite. It usually

produces chitinases to break down the walls of its host. If the pathogen is an oomycete, then cellulases are needed by the antagonist.

Trichoderma is perhaps the best known mycoparasite (Papavisas, 1985; Haran et al., 1996). It is a proven biological control agent against many soil pathogens and is one of the few agents at present commercially available (Elad et al., 1993). *Trichoderma* hyphae may penetrate resting structures such as sclerotia or may parasitize growing hyphae. The hyphae grows alongside the host and sends out side branches that coil around the host hypha. Penetration of the wall has been shown in some cases.

1.5.5 Fungistasis

Fungistasis is the prevention of fungal growth, mainly by carbon limitation (Lockwood, 1986). May pathogens in the soil produce resting structures of various kinds that remain dormant in the soil until nutrients are available. Fungistasis is imposed on the pathogen when the saprotrophic microflora makes use of the available carbon; germination and subsequent infection is prevented. One of the best examples of this is in soils where the competition for carbon amongst *Fusarium* species leads to a reduction of the disease.

Figure 1.1. Black rot of pineapple fruit.caused by the fungus *Chalara paradoxa* (De Seyn.) Sacc. = *Thielaviopsis paradoxa* (De Seyn.) Hohn., teleomorph: *Ceratocystis paradoxa.* The disease starts in the field where the fungus typically enters the core through the broken peduncle of the fruit and advances upwards as a cone, progressing along the core more rapidly than through the flesh. The fungus may also enter through natural growth cracks or wounds on the fruit shell.

Figure 1.2. Flesh appearance of pineapple fruit with black rot. Fruit tissue has a water-soaked appearance and becomes dark yellow. The fruit is very soft and juicy, even in the early stages of decay, and the flesh becomes so thoroughly disintegrated that it yields to the slightest pressure.

Figure 1.3. Black spore formation that takes place over the cut surface of diseased tissue when exposed to air for 24 hr. This black formation consists of the dark colored macrospores.

CHAPTER 2

SIGNIFICANCE OF STUDY, HYPOTHESES AND OBJECTIVES

The use of postharvest chemicals, which includes fungicides, fumigants, sprout suppressants, and antioxidants, has resulted in the extension of the storage life and shelf life of fruits and vegetables. A broader geographic area from which produce is sourced and an all-year-round supply of many perishable commodities to the consumer has also been realized. However, the use of these chemicals are under threat due to a number of reasons, one of which is the increased pressure from the public to limit the use of pesticides on produce (Ragsdale and Sisler,, 1994). This pressure is caused by an increased awareness of the presence of pesticide residues and their possible carcinogenic effect. There is therefore a need for alternative methods to control postharvest diseases in fruits and vegetables. Biological control using antagonistic microorganisms is one of the promising options.

Factors that affect the potential for biological control include: environmental conditions, the profile of microorganisms on the fruit during growth and the influence of postharvest handling on these organisms, and which components of that microbial profile make the fruit more susceptible or resistant to disease.

The hypotheses tested in this study were:

1. The microbial population on the pineapple fruit shell is dependent upon preharvest weather parameters.
2. Changes in the microbial population on the pineapple fruit shell influence the incidence and severity of pineapple black rot.

3. Washing the pineapple fruit results in a decrease in the beneficial microbial population on the fruit shell.
4. A decrease in the beneficial microbial population on the pineapple fruit shell increases the incidence and severity of black rot.
5. Filamentous fungi and yeasts found on the pineapple fruit shell offer the fruit protection against the black rot pathogen, *Chalara paradoxa*.
6. Use of yeast antagonist(s) effective against *Chalara paradoxa* is compatible with current industry practice of keeping pineapple fruit at low temperature (8 - 10°C) during transport.
7. Combining yeast antagonist(s) with a low dose of Bayleton results in a reduction of incidence and severity of black rot in pineapple fruit comparable to the level of control achieved with the commercial dose of Bayleton used by the industry.
8. In the presence of a yeast antagonist, *Chalara paradoxa* growth is inhibited.

To test the above hypotheses, the study had the following objectives:

1. Monitor microbial growth on the pineapple fruit shell for a period of over a year and correlate the population dynamics with the incidence and severity of black rot and weather parameters during fruit growth.
2. Determine if certain steps in the fruit handling system affect the fruit microbial population that would predispose fruit to more disease.
3. Isolate microbial antagonist(s) from the pineapple fruit shell and test against the plant pathogen *Chalara paradoxa*.

4. Determine if an isolated antagonist is compatible with current industry practice of keeping pineapple fruit at low temperature (8 - 10°C) during transport.

5. Determine if combining yeast antagonist(s) with a low dose of Bayleton results in a reduction in the incidence and severity of black rot in pineapple fruit comparable to the level of control achieved with a commercial dose of Bayleton.

6. Determine whether *Chalara paradoxa* spore germination and germ tube growth is inhibited in the presence of yeast antagonists.

CHAPTER 3

RELATIONSHIPS AMONG MICROBIAL POPULATION ON PINEAPPLE FRUIT SHELL, BLACK ROT INCIDENCE AND SEVERITY AND WEATHER PARAMETERS

3.1 Introduction

In order to initiate a successful biological control program, fundamental information must be known on the nature of the relationship between the host, pathogen and associated microflora. Such a relationship is influenced by external factors such as the microclimate at the plant surface and seasonal weather changes which cause the plant surface microhabitat to be in a state of continuous fluctuations.

It is widely accepted that biological control occurs naturally on plant surfaces and that antagonistic activity of saprophytic microflora against pathogens reduces incidence of disease. This is the premise on which most work on biological control of pathogens starts. A search is conducted for potential antagonists in the habitat in which pathogen is normally found. This search includes an assessment of the microbial population in that habitat, both in terms of species composition and propagule number. Once the species composition of a habitat has been established, the next step is to carry out screening of potential antagonists.

The objectives of this study were the following: 1) Monitor microbial growth on the pineapple fruit shell for more than one year and correlate the population dynamics with the incidence and severity of black rot and weather parameters during fruit growth, 2) Determine if certain steps in the fruit handling system affect the fruit microbial population that would predispose fruit to more disease, and 3) Isolate and identify microorganisms on the pineapple fruit shell.

3.2 Materials and methods

3.2.1 Fruit samples

Pineapple fruit (*Ananas comosus* cv. Smooth Cayenne, Champaka F-153) were obtained from Dole Fresh Fruit Co. at Wahiawa, Hawaii on the island of Oahu. Fruit obtained were placed in plastic crates and transported within 2 hr to the laboratory on the University of Hawaii, Manoa campus. Fruit were either used immediately or held at room temperature (22°C) and experiments installed within 24 to 48 hr.

3.2.2 Fruit evaluation

3.2.2.1 Shell color

Fruit shell color was evaluated according to the Dole Fresh Fruit Color Standards: 0 - full green, 1 - slight color break, 2 - less than ¼ yellow, 3 - ¼ to ½ yellow, 4- ½ to ¾ yellow, 5 - ¾ to full yellow, 6 - full yellow to ¼ reddish brown, and 7 - more than ¼ reddish brown (Figure 3.1).

3.2.2.2 Black rot

Individual fruit were cut lengthwise and evaluated for incidence and severity of black rot. Incidence of black rot was evaluated as percentage of the total number of fruit infected. Severity of black rot was evaluated by estimating the percentage of surface area that is diseased.

3.2.3 Microbial population monitoring

Fruit at shell color 1 to 2 were obtained on alternate weeks for 14 months at two points in the handling system: 1) in the field, and, 2) on the culling line. Field-sampled fruit, designated as dry fruit, were taken from the slide chute just before the fruit dropped into the

wooden field bins atop a truck that transported it to the packing plant. Fruit from the same field bin were followed to the packing plant and sampled at the culling line. Fruit were taken from the roller conveyor just after fruit had been floated out of the field bins into a water bath and elevated to the culling station; these fruit were designated as wet fruit. Just before fruit reach the culling line, fruit were subjected to a clean water spray.

Twenty fruit were taken at each sampling point, ten fruit were washed for isolation of microorganisms and the remaining ten fruit were kept at room temperature (22°C) for one week, then evaluated for incidence and severity of black rot. Occasionally, due to unforseen circumstances, sampling had to be modified. On one occasion, 40 fruit had to be harvested in the field, transported to the packing plant, tagged individually, dipped in the floating tank and then retrieved on the culling line. Several times, dry fruit samples had to be obtained in field bins that had already been transported to the packing plant. This was mainly due to rain, and the muddy and unsafe driving conditions in the pineapple fields.

Almost halfway through the experiment, Dole Fresh Fruit Co. changed their handling operations to in-field packing of the fruit, thus eliminating fruit washing. Sampling for the experiment was modified and only dry fruit samples were obtained. Initially, fruit were sampled in the field alongside the pickers just before they dipped the fruit in the wax-Bayleton bucket. Eventually, sampling was done by harvesting fruit from the same field that the in-field packing equipment was located. This was done to ensure that fruit obtained was at commercial maturity.

3.2.4 Isolations

Microbial isolations were done by washing individual fruit in sterile jars with 400 ml of sterile distilled water on a rotary shaker at 100 rpm for 10 minutes. Serial dilutions of the wash water were plated out on potato dextrose agar (PDA) and WORT agar (Difco; medium for enumerating and cultivating yeasts) to determine the dilution for microbial counting. Eventually plating was done on WORT agar only, as it gave more kinds of isolates. Plates were incubated at room temperature (22°C), observed daily and colonies counted after seven days.

3.2.5 Identification of isolates

Filamentous fungi were identified by microscopic examination. Yeast isolates were identified using the BIOLOG system of identification (Biolog, Inc., Hayward, CA). Biolog Microplates containing 96 wells were used to test the ability of the isolate to utilize or oxidize a preselected panel of different carbon sources. The test yielded a characteristic pattern of purple wells that constituted a "metabolic fingerprint" of the capacities of the inoculated isolate.

3.2.6 Weather data

Dole Fresh Fruit Co. does not monitor weather parameters in their fields. Weather data which included rainfall, minimum and maximum temperatures, and solar radiation, were obtained from the Poamoho Experiment Station of the College of Tropical Agriculture and Human Resources. The weather data obtained from this station is part of the data base of the National Oceanic and Atmospheric Administration. The experiment station was located

within 10 km of all harvested pineapple fields. Elevation of the area ranged from 545 to 705 ft above sea level.

3.2.7 Experimental setup and data analysis

Experiments were setup in a completely randomized design with ten replications per treatment. Individual fruit served as a replicate. Platings on WORT agar for microbial isolations were done on three plates per replicate. Correlation analysis (Statistical Analysis Systems Institute Inc., Cary, North Carolina) was used to determine the possible relationships among microbial, filamentous fungi, and yeast populations on pineapple fruit shell, black rot incidence and severity, and weather parameters.

3.3 Results

Variation in total microbial population on the pineapple fruit shell taken from May 1996 to June 1997 are shown in Figure 3.2A. Microbial counts had low variability from month to month except in November when rainfall was high (Figure 3.2D). Filamentous fungi and yeasts were the principal types of microbes in the total microbial population (Figure 3.2B), from various genera (Table 3.1). There are a number of filamentous fungi and yeast isolates that were unidentified. Black rot incidence and severity was highest during the months of August to November, however, severity of the disease reached only 9% (Figure 3.2C). Rainfall was scattered throughout the 14 month period with a peak in November (Figure 3.2D). Temperature did not vary greatly, ranging from a minimum of 18.2° to a maximum of 30.8°C(Figure 3.2E).

Correlation analysis showed no correlation between black rot incidence or severity and total microbial, filamentous fungi and yeast population on pineapple fruit shell, except for

the significant correlation between black rot incidence of wet fruit and filamentous fungi population on wet fruit (Table 3.2). Black rot incidence and severity of dry fruit was not correlated with rainfall in the month of harvest up to four months before harvest (Table 3.3). Black rot incidence of dry fruit was positively correlated to maximum and minimum temperature one, two, and three months before harvest (Table 3.3). Black rot severity of dry fruit was positively correlated with maximum temperature in the month of harvest, one and two months before harvest and minimum temperature one and two months before harvest (Table 3.3).

Correlation analysis of microbial counts and weather parameters are presented in Table 3.4. The total microbial population of dry pineapple fruit shell was positively correlated with rainfall in the month of harvest and maximum temperature one month before harvest. No correlation was found between total microbial population of wet fruit and the weather parameters. Filamentous fungi population of dry fruit was positively correlated with rainfall four months before harvest and negatively correlated with minimum temperature three months before harvest. On wet fruit, filamentous fungi population was negatively correlated with maximum temperature one month before harvest, minimum temperature three months before harvest, and positively correlated with solar radiation two months before harvest. Yeast population of dry fruit was negatively correlated with rainfall three months before harvest. On wet fruit, yeast population was negatively correlated with maximum temperature one month before harvest and minimum temperature three months before harvest.

Table 3.1. Genera of filamentous fungal and yeast isolates on the pineapple fruit shell.

Filamentous fungi	Yeast
Acremonium	*Candida*
Cephalosporium	*Cryptococcus albidus*
Cladosporium	*Cryptococcus*
Fusarium	*Pichia guilliermondii*
Geotrichum	*Rhodoturula aurantiaca*
Gliocladium	*Rhodoturula glutinis*
Mortierella	
Penicillium	

Table 3.2. Pearson correlation coefficients between pineapple black rot incidence and severity and total microbial count, filamentous fungi count, yeast count on pineapple fruit shell.

Fruit parameter/ Weather period	Total microbial count		Filamentous fungi count		Yeast count	
	dry	wet	dry	wet	dry	wet
Black rot incidence, dry fruit	0.489		-0.371		0.002	
Black rot incidence, wet fruit		-0.722		-0.910*		-0.790
Black rot severity, dry fruit	0.429		-0.361		0.057	
Black rot severity, wet fruit		-0.681		-0.850		-0.713

* Coefficient significant at $P < 0.05$.

Table 3.3. Pearson correlation coefficients between pineapple black rot incidence and severity on unwashed fruit and weather parameters.

Fruit parameter/ Weather period	Rainfall	Maximum temperature	Minimum temperature	Solar radiation
Incidence				
Month of harvest	0.424	0.419	0.387	-0.298
1 month before harvest	0.078	0.678**	0.677**	-0.088
2 months before harvest	-0.167	0.672**	0.749**	-0.028
3 months before harvest	-0.191	0.650*	0.672**	0.132
4 months before harvest	-0.317	0.448	0.382	0.535
Severity				
Month of harvest	0.317	0.562*	0.504	-0.179
1 month before harvest	-0.073	0.681**	0.663*	0.046
2 months before harvest	-0.178	0.625*	0.707**	-0.027
3 months before harvest	-0.193	0.518	0.538	0.076
4 months before harvest	-0.325	0.314	0.269	0.493

*, **, Coefficient significant at $P < 0.05$ and 0.01, respectively.

Table 3.4. Pearson correlation coefficients between total microbial count, filamentous fungi count, yeast count on pineapple fruit shell and weather parameters.

Weather period	Rainfall	Max temp	Min temp	Solar radiation
Total microbial count, dry fruit				
Month of harvest	0.650 *	0.323	0.421	-0.348
1 month before harvest	-0.460	0.619*	0.531	0.272
2 months before harvest	-0.204	0.427	0.443	0.348
3 months before harvest	-0.383	0.323	0.095	0.394
4 months before harvest	0.192	-0.405	-0.463	0.032
Total microbial count, wet fruit				
Month of harvest	0.060	-0.252	-0.164	-0.935
1 month before harvest	-0.364	-0.872	-0.592	-0.141
2 months before harvest	-0.531	0.232	-0.040	0.773
3 months before harvest	0.442	-0.593	-0.770	0.471
4 months before harvest	0.483	-0.829	-0.769	-0.497
Filamentous fungi count, dry fruit				
Month of harvest	0.041	-0.019	-0.070	-0.057
1 month before harvest	-0.345	-0.076	-0.166	0.216
2 months before harvest	-0.011	-0.144	-0.200	0.316
3 months before harvest	-0.475	-0.368	-0.636*	0.309
4 months before harvest	0.569*	-0.534	-0.508	-0.425

Table 3.4. (Continued)Pearson correlation coefficients between total microbial count, filamentous fungi count, yeast count on pineapple fruit shell and weather parameters.

Weather period	Rainfall	Max temp	Min temp	Solar radiation
Filamentous fungi count, wet fruit				
Month of harvest	-0.358	-0.514	-0.571	-0.709
1 month before harvest	-0.316	-0.999***	-0.849	0.808
2 months before harvest	-0.185	-0.249	-0.512	0.978*
3 months before harvest	0.376	-0.842	-0.960**	0.178
4 months before harvest	0.749	-0.678	-0.747	-0.655
Yeast count, dry fruit				
Month of harvest	-0.117	0.542	0.405	0.114
1 month before harvest	-0.448	0.374	0.384	0.361
2 months before harvest	-0.249	0.323	0.247	0.372
3 months before harvest	-0.603*	-0.159	-0.420	0.525
4 months before harvest	0.293	-0.361	-0.395	0.018
Yeast count, wet fruit				
Month of harvest	-0.271	-0.346	-0.407	-0.771
1 month before harvest	-0.433	-0.970*	-0.815	-0.147
2 months before harvest	-0.183	0.055	-0.237	0.848
3 months before harvest	0.374	-0.802	-0.910*	0.235
4 months before harvest	0.573	-0.637	-0.662	-0.591

*, **, *** Coefficient significant at $P < 0.05$, 0.01, and 0.001 respectively.

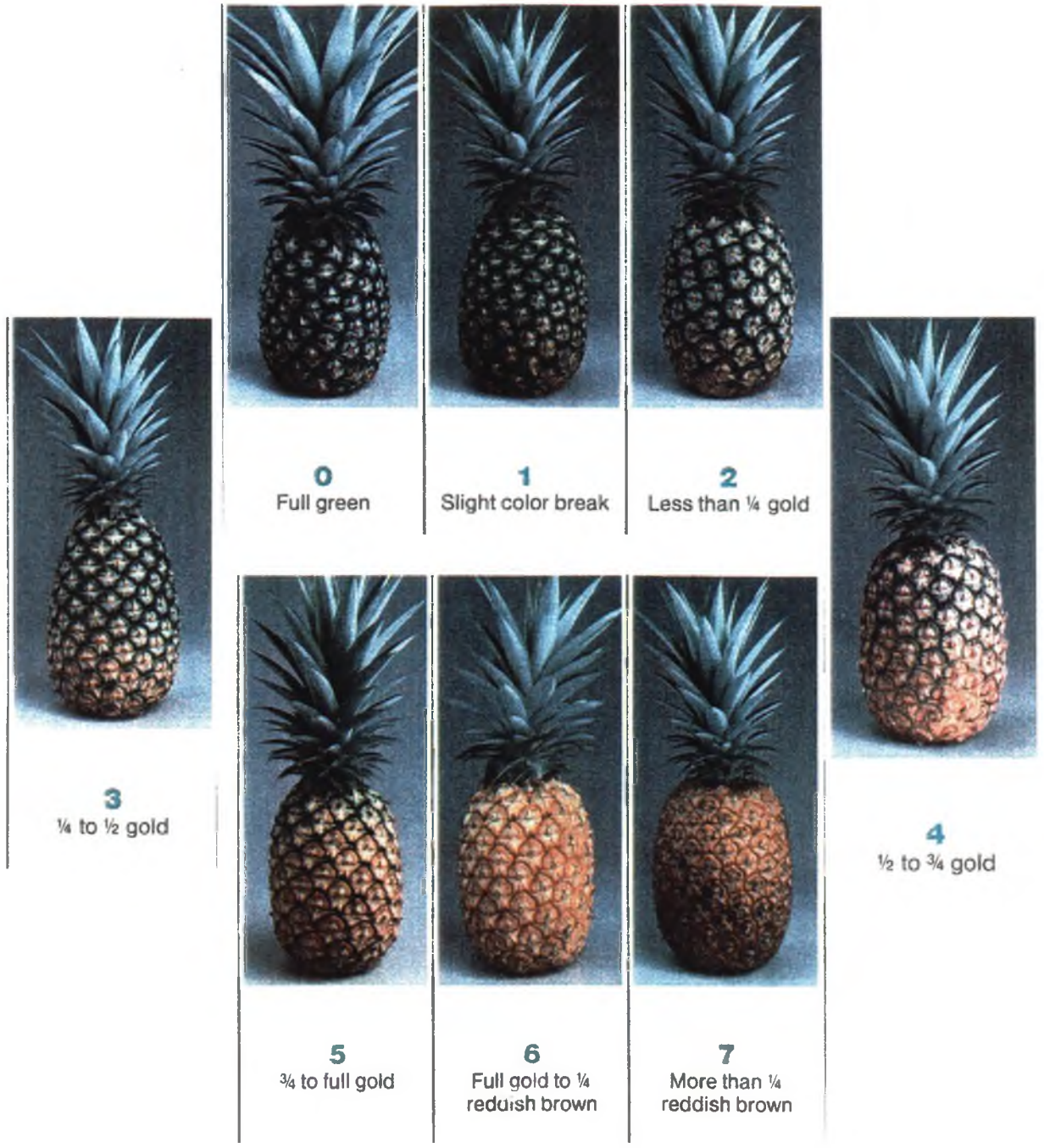

Figure 3.1. Dole Fresh Pineapple Color Standards Guide.

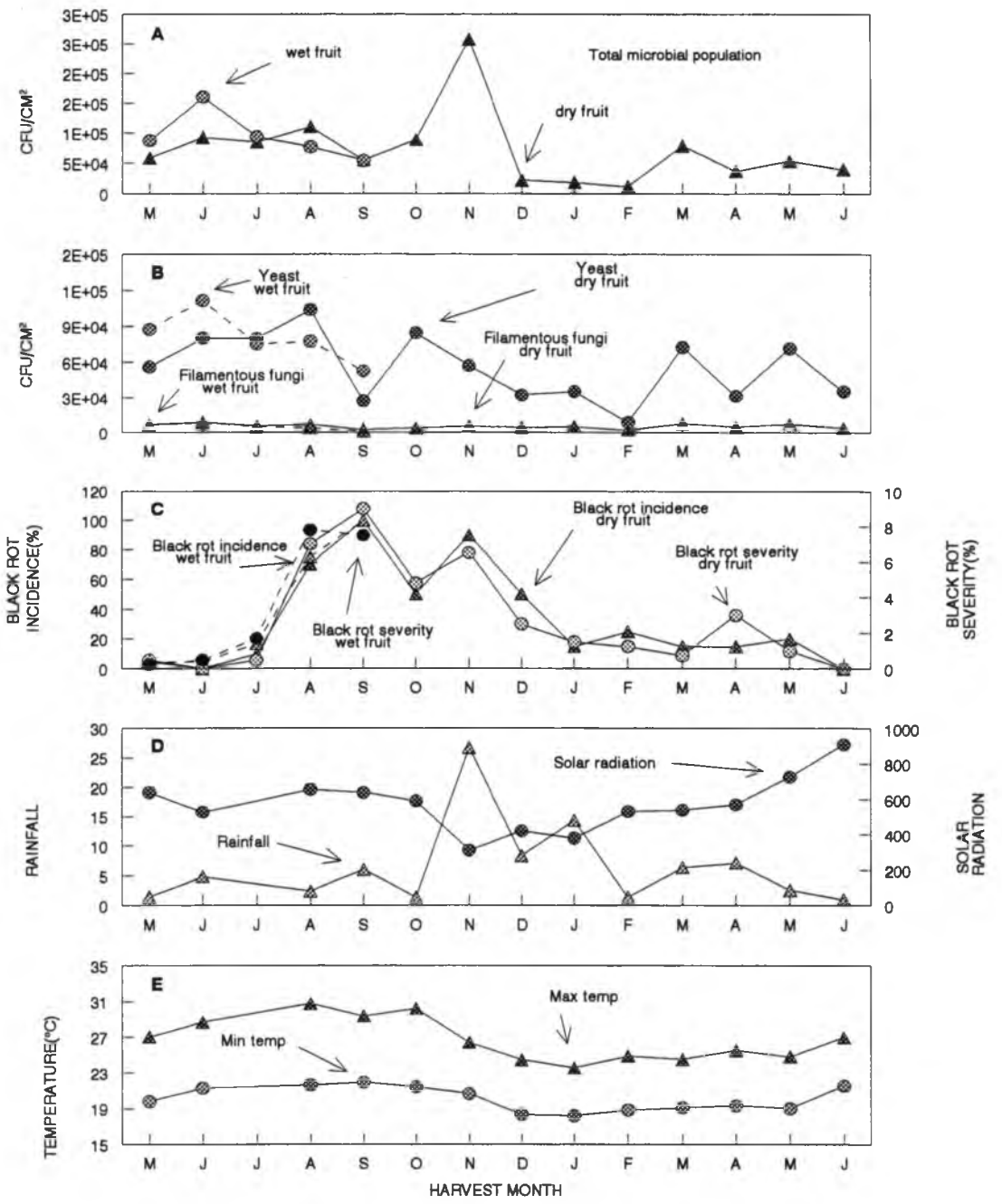

Figure 3.2. Total microbial count as colony forming units cm^{-2} (CFU/cm^2) on pineapple fruit shell from May 1996 to June1997 (A), fungal and yeast counts on pineapple fruit shell (B), black rot incidence and severity (C), variation in total rainfall and solar radiation (D), and maximum and minimum temperatures (E) for the same period.

3.4 Discussion

Plant surfaces are colonized by diverse microbial flora. The filamentous fungal and yeast isolates from the pineapple fruit shell (Table 3.1) occur on other plant species. *Acremonium* is a potential biocontrol agent for apple decay (Janisiewicz, 1987) and *Cladosporium* has been found on strawberry (Lima et al., 1997) and peach bark (Buck et al., 1998). *Fusarium* occurs on pineapple, (Rohrbach and Taniguchi, 1984) and in watermelon (Larkin et al., 1996). *Penicillium* can be found in strawberry (Lima et al., 1997), causes rots in apple (McLaughlin et al., 1992), and causes a number of diseases in pineapple (Rohrbach and Pfeiffer, 1976b; Rohrbach and Taniguchi, 1984). *Gliocladium* is a biocontrol agent (Papavisas, 1985) and it occurs in nearly all soils and other natural habitats, especially in those containing or consisting of organic matter. It has also been reported on strawberry (Peng and Sutton, 1991). *Candida* can be found on lemon (Wilson and Chalutz, 1989), *Cryptococcus* on wheat leaves (Dik et al., 1992), peach bark (Buck et al., 1998), pear fruit (Chand-Goyal and Spotts, 1996), and olives (Castoria et al., 1997) and *Rhodoturula* is found on peach bark (Buck et al., 1998), pear fruit (Chand-Goyal and Spotts, 1996), and olives (Castoria et al., 1997). *Pichia* found on lemon is a biocontrol agent against postharvest diseases in citrus fruit (Droby et al., 1993).

Yeasts were the majority of the microbial population on the pineapple fruit shell (Figure 3.2B) with the remainder being filamentous fungi. Similar results have been shown for peach bark where yeasts and yeast-like fungi dominated the mycoflora during potential infection periods in the spring and fall (Buck et al., 1998) and on European larch leaves (McBride and Hayes, 1977). It is possible that the yeasts dominated over the other

microorganisms because they produce extracellular polysaccharides that enhance their survivability, which may restrict colonization sites and the flow of germination cues to fungal propagules (Janisiewicz, 1988a). Furthermore, the yeasts rapidly use available nutrients and proliferate (Janisiewicz, 1988b). Some yeast isolates appeared similar to the descriptions of pineapple yeasts isolated and previously reported by the Pineapple Research Institute (PRI) in the 1940s (Okimoto, n.d., Anonymous, 1940).

Counts of individual isolates were attempted to determine whether changes in the filamentous fungal and yeast populations correlated with black rot incidence and severity in pineapple fruit. Unfortunately, exact counts could not be made as some isolates that were thought to be the same, later turned out to be two, or three different species when pure cultures were grown on PDA plates. Therefore, counts for filamentous fungi and yeast were pooled separately. The population of filamentous fungi fluctuated only slightly during the period of observation, but not as much as the yeast population (Figure 3.2B). Populations of yeasts and filamentous fungi isolated from peach bark were highest in the fall, dropped to low levels in the winter, and increased in the spring (Buck et al., 1998). A greater diversity of fungi was present on the bark surfaces in the fall than in the spring. Melgarejo and co-workers (1985) reported increased fungal diversity on peach twigs and flowers in spring and summer. These published results suggest that the microbial population and diversity found on peach bark, twigs, and flowers are affected by changes in weather conditions throughout the growing season. In the present study, there was no pronounced trend seen in the microbial populations with respect to the season (wet vs. dry). Although, a greater microbial population was observed when rainfall was high in the month of harvest (Figure 3.2). Total

microbial population on dry fruit correlated (P = 0.05) to rainfall in the month of harvest, which was to be expected as there were greater chances for the fruit being splashed by water and soil, a rich source of microorganisms. The filamentous fungi population on dry fruit was negatively correlated with minimum temperature three months before harvest (Table 3.4). The fairly high correlations seen in filamentous fungi and yeast population of wet fruit with maximum and minimum temperatures (Table 3.4) seemed to be coincidental, as temperature would not be expected to be a factor in the growth of the microorganisms on the fruit just because it was washed. However, the temperature may influence the recolonization rate if one of the microorganisms is closer to its optimum temperature of growth.

Pink (*Rhodoturula sp.*) and white yeasts (*Pichia sp.* and *Cryptococcus sp.*) were isolated from the pineapple fruit shell (Table 3.1). Although exact counts were not made, it was observed that white yeasts occurred more frequently and in greater numbers than pink yeasts. It has been reported that pink and white yeasts (*Sporobolomyces roseus* and *Cryptococcus laurentii*, respectively) on wheat leaves can grow at temperatures ranging from 12° to 24°C (Dik et al., 1992). The optimum growth temperature for the white yeasts was slightly higher than for pink yeasts if the relative humidity alternated between 70 and 95%. This was offered as an explanation for the increase in relative occurrence of white yeasts later in the season (Dickinson and Wallace, 1976). This may also be a possible explanation for the observation made in the current study on pineapple fruit. Furthermore, *Cryptococcus laurentii* (Dik et al., 1992) and *Sporobolomyces roseus* (Bashi and Fokkema, 1977) populations increased rapidly with small vapor pressure deficits. Apparently, when the vapor

pressure deficit is small during part of the day, free water on the phyllosphere was not necessary for yeast population growth.

Black rot incidence and severity was highest during the months of August to November, however, disease severity reached only 9% (Figure 3.2C). Generally, no relationship can be seen between black rot incidence or severity and changes in total microbial, filamentous fungi, yeast populations, except for the significant, negative correlation between black rot incidence of wet fruit and filamentous fungal population of wet fruit (Table 3.2). Similar results have been reported for banana crown rot incidence and changes in the occurrence of causal organisms present on the crown (Lukezic et al., 1967) or changes in fungal populations (Wallbridge, 1981). Wallbridge (1981) suggested that difficulty in sampling, presence of moribund hyphal fragments in advance stages of rot, synergistic effects between fungal species and between fungal species and bacteria could have the reason why there was no correlation found between banana crown rot incidence and changes in fungal populations. In the present study, this can all be true especially the difficulty in sampling. The manner of sampling had to be changed several times due to the change of operations of Dole Fresh Fruit Co. and also due to unfavorable weather conditions that did not permit direct field sampling.

The negative correlation between black rot incidence of wet fruit (fruit that had been washed) and filamentous fungal population of wet fruit (Table 3.2) suggests that washing the fruit removed some of the beneficial filamentous fungi on the pineapple fruit shell, predisposing the fruit to black rot. Washing fruit and vegetables has been shown to increase

susceptibility to decay (Wilson and Wisniewski, 1989) due partially to the removal of disease-suppressing antagonistic organisms.

Rainfall did not appear to be a major factor in black rot incidence and severity. Black rot incidence was highly correlated with maximum and minimum temperatures one, two, and three months before harvest (Table 3.3). Black rot severity was correlated with maximum temperature in the month of harvest, one and two months before harvest and minimum temperature, one and two months before harvest. These correlations between black rot incidence and severity with maximum and minimum temperatures may be due indirectly to the effect of temperature on the microbial growth on the pineapple fruit shell. However, this does not seem to be the case since there was little correlation between total microbial, filamentous fungi, and yeast populations with maximum and minimum temperatures (Table 3.4).

Due to the change in operations in Dole Fresh Fruit, determination of whether washing conclusively affected microbial population on the pineapple fruit, incidence and severity of black rot was not completed.

CHAPTER 4

SCREENING POTENTIAL ANTAGONISTS

4.1 Introduction

There are two systems for screening potential antagonists: *in vitro* tests using laboratory cultures and *in vivo* tests using the whole plant, whole leaf, or whole fruit.

Results of *in vitro* tests may be initially encouraging as they give clear and visible results in antagonist tests on plates. They are relatively easy to perform and it lends itself to screening a large number of isolates. However, they select for antibiotic producers and are poor predictors of the activity of the organism in the field. The decision to use this method is often based upon space limitations, absence of host plants and a means of growing them in glasshouses, along with limited time and staff.

In vivo tests are the choice of most researchers as they most closely imitate the conditions under which the control agent will eventually have to operate. The amount of disease control is measured regardless of mode of action. The overwhelming problem with these tests is the time, effort and money necessary.

The objectives of this study were: 1) Determine whether pineapple fruit wash water contained possible antagonists, thereby giving the pineapple fruit protection against the plant pathogen *Chalara paradoxa.* This was achieved by initial screening using the selection strategy of Wilson et al. (1993); and 2) Screen, *in vitro*, the most frequently occurring microbial isolates obtained from the pineapple fruit shell against *Chalara paradoxa*.

4.2 Materials and methods

4.2.1 Wash water as possible source of antagonists

4.2.1.1 Fruit handling and evaluation

Fruit with shell color 1 or 2 (Figure 3.1) were harvested from field rows beyond the third row from the road to avoid dust contamination. The peduncle was cut with a knife or pruning shears so as to leave 7.5 to 10 cm of peduncle on the fruit, to prevent possible contamination of the fruit with field inoculum. Fruit were laid down in clean baskets and transported to the laboratory. The peduncle was broken off the fruit by hand just before inoculation. All inoculations were done by atomizing one ml of prepared spore suspensions on the cut peduncle end of the pineapple fruit. Fruit were evaluated as in 3.2.2.

4.2.1.2 Inoculations

Fruit were washed in the same manner that was used to determine microbial population on the pineapple fruit shell (as in 3.2.4) . This wash water was used to inoculate fruit to determine if it contained beneficial microorganisms that can protect the fruit from *C. paradoxa*. One ml of wash water was atomized on the cut peduncle end of the pineapple fruit. The cut peduncle end of the fruit was chosen as the infection court instead of the whole fruit since it was easier to control. If the whole fruit was chosen as the infection court, it would have been more difficult to assess incidence and severity of black rot since the fruit shell of the pineapple has a lot of growth cracks or natural openings which can complicate evaluation of disease. After 2 hr, fruit were inoculated with a one ml spore suspension (1 x 10^6 spore ml^{-1}) of *C. paradoxa*. Control fruit were sprayed with sterile distilled water. Fruit

were kept at room temperature (22°C) in open shelves or baskets for 7 days, then evaluated for incidence and severity of black rot and shell color.

4.2.2 *In vitro* screening

4.2.2.1. Cultures

Cultures of filamentous fungal isolates were maintained on potato dextrose agar (PDA) slants at 4°C. A spore suspension of 1 x 10^6 spores ml^{-1} was prepared from 7 day old cultures grown on PDA at 27°C. Cultures were flooded with sterile distilled water and the surface of the culture was carefully scraped with a sterile, disposable loop without disturbing the agar. Spore concentration was determined with a hemacytometer and suspensions were used within 2 to 3 hr.

Yeast isolates obtained from the pineapple fruit shell were maintained on malt extract agar (MEA) slants at 4°C. A spore suspension of 1 x 10^8 spores ml^{-1} was prepared from 48 hr old cultures grown on MEA plates at 27°C. Cultures were flooded with sterile distilled water and the surface of the culture was carefully scraped with a sterile, disposable loop without disturbing the agar. The resulting suspension was vortexed for ca. 30 sec to break up any chains of spores. Concentrations were determined with a hemacytometer and suspensions were used within 2 to 3 hr.

4.2.2.2. Screening

Microbial isolates were initially screened for potential *in vitro* antagonistic activity against *Chalara paradoxa*. Initially, the most frequently isolated filamentous fungi, then the fastest growing filamentous fungal isolates were chosen and used in antagonist tests against *C. paradoxa* on glucose yeast extract agar (GYEA: 3 g glucose, 1 g yeast extract, 20 g agar

1 l distilled water) plates. Two methods were used: 1) mixture of spores of the potential antagonist and *C. paradoxa,* and, 2) 4 mm discs of each of the potential antagonists and *C. paradoxa*. The most frequently isolated yeasts were then screened on the same medium. A disc of *C. paradoxa* was placed in the center of the plate and aliquots of different concentrations of the yeast isolates were placed at the plate margins. Plates were incubated at room temperature and evaluated after 7 days. Evaluation was done using the following scale: 1 = complete inhibition, 2 = partial inhibition, and 3 = no inhibition, overgrown with *C. paradoxa*. The level of inhibition was designated as complete, when there was no *C. paradoxa* growth on the yeast colony; partial, when sparse sporulation of *C. paradoxa* was observed on the margin of the yeast colony; and no inhibition, when the yeast colony is overgrown with *C. paradoxa*.

4.2.3 Experimental setup and data analysis

Experiments were setup in a completely randomized design with twenty replications per treatment, unless otherwise noted in the data tables. Individual fruit or plate served as a replicate. All experiments were repeated once. Statistical analyses were done using the general linear models procedure (Statistical Analysis Systems Institute Inc., Cary, North Carolina) with mean separation with Waller-Duncan.

4.3 Results

Pineapple fruit inoculated with *Chalara paradoxa* had significantly less severe black rot when atomized with wash water from the pineapple fruit shell compared to spraying with sterile distilled water (Tables 4.1 and 4.2, Figures 4.1 and 4.3). Fruit shell color was consistently higher in fruit inoculated with *C. paradoxa* compared to the control fruit (Tables

4.1 and 4.2, Figure 4.2). Black rot incidence was 100% in fruit inoculated with *C. paradoxa* (Tables 4.1 and 4.2). Fruit that had been washed in sterile distilled water without inoculation with *C. paradoxa* developed rot, while unwashed fruit not inoculated with *C. paradoxa* did not develop rot (Table 4.1, Figure 4.1). A longer interval between atomizing the wash water on the cut peduncle end of the pineapple fruit and atomizing with a spore suspension of *C. paradoxa* resulted in a significant reduction of black rot severity (Table 4.2, Figure 4.3). All the frequently isolated filamentous fungal isolates (Table 4.3), including a fast growing one were overgrown with *C. paradoxa* when grown together in glucose yeast extract agar (GYEA) plates. On the other hand, yeast isolates that were screened for antagonistic activity against *C. paradoxa* were able to inhibit the growth of the pathogen when grown on GYEA plates (Table 4.4 and Figure 4.4).

Table 4.1. Black rot incidence and severity and shell color of pineapple fruit inoculated by atomizing with pineapple fruit wash water and *Chalara paradoxa*.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]	Shell color [d]
Unwashed control	0 c	0 d	2 b
Washed control	75 b	4 c	2 b
Sterile distilled H_2O + CP, 2 hr later	100 a	38 a	4 a
Wash H_2O + CP, 2 hr later	100 a	36 b	4 a
Analysis of variance Pr > F	0.0001	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 20).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that is diseased.

[d] Fruit shell color was evaluated according to the Dole Fresh Fruit Color Standards: 0 - full green, 1 - slight color break, 2 - less than ¼ yellow, 3 - ¼ to ½ yellow, 4- ½ to ¾ yellow, 5 - ¾ to full yellow, 6 - full yellow to ¼ reddish brown, and 7 - more than ¼ reddish brown.

Table 4.2. Black rot incidence and severity and shell color of pineapple fruit inoculated by atomizing with pineapple fruit wash water and *Chalara paradoxa* at different time intervals.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]	Shell color [d]
Sterile distilled H_2O	0 b	0 e	3.6 d
Sterile distilled H_2O + CP, 2 hr later	100 a	39 b	4.8 bc
Wash H_2O + CP, 2 hr later	100 a	44 a	5.0 ab
Wash H_2O + CP, 8 hr later	100 a	30 c	5.2 a
Wash H_2O + CP, 16 hr later	100 a	24 d	4.8 bc
Wash H_2O + CP, 24 hr later	100 a	21 d	4.6 c
Analysis of variance Pr > F	0.0001	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 20).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that is diseased.

[d] Fruit shell color was evaluated according to the Dole Fresh Fruit Color Standards: 0 - full green, 1 - slight color break, 2 - less than ¼ yellow, 3 - ¼ to ½ yellow, 4- ½ to ¾ yellow, 5 - ¾ to full yellow, 6 - full yellow to ¼ reddish brown, and 7 - more than ¼ reddish brown.

Table 4.3. Screening of most frequently isolated filamentous fungal isolates from pineapple fruit shell against *Chalara paradoxa* (CP) on glucose yeast extract agar (GYEA) plates. Plates were held at 22°C for 7 days then evaluated.

Isolate	Spore mixture	Discs
Fusarium	overgrown with CP	overgrown with CP
Penicillium 1	overgrown with CP	overgrown with CP
Penicillium 2	overgrown with CP	had extensive mycelial growth, but overgrown with CP
Mortierella	overgrown with CP	overgrown with CP
Acremonium	overgrown with CP	overgrown with CP
Gliocladium	had extensive mycelial growth, but overgrown with CP	overgrown with CP
Unidentified isolate - fast growing	-	overgrown with CP

Table 4.4. Screening of most frequently isolated yeast isolates *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1), and *Cryptococcus sp.* (Cryptococcus) from pineapple fruit shell, at different inoculum concentrations, against *Chalara paradoxa* (CP) on glucose yeast extract agar (GYEA) plates.[a] Plates were held at 22°C for 7 days then evaluated.

Yeast antagonist	Inoculum concentration[b]/ Level of control[cd]			
	1/4x	1/2x	1x	2x
WFO	1 c	1 c	1 c	1 c
Old	1 c	1 c	1 c	1 c
SWC	1.7 a	1.4 b	1.5 ab	1.3 b
Analysis of variance Pr > F		0.0001		

[a] A disc of *C. paradoxa* was placed in the center of the plate and aliquots of different concentrations of the yeast isolate were placed at the plate margins.

[b] Inoculum concentrations (1x) used were as follows: Pichia, 2.6×10^8 spores ml^{-1}; Rhodoturula1, 1.6×10^8 spores ml^{-1}; Cryptococcus, 2.3×10^8 spores ml^{-1}.

[c] Evaluated using the following scale: 1 = complete inhibition, 2 = partial inhibition, and 3 = no inhibition, overgrown with CP.

[d] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column and row followed by the same letter were not significantly different (n = 10).

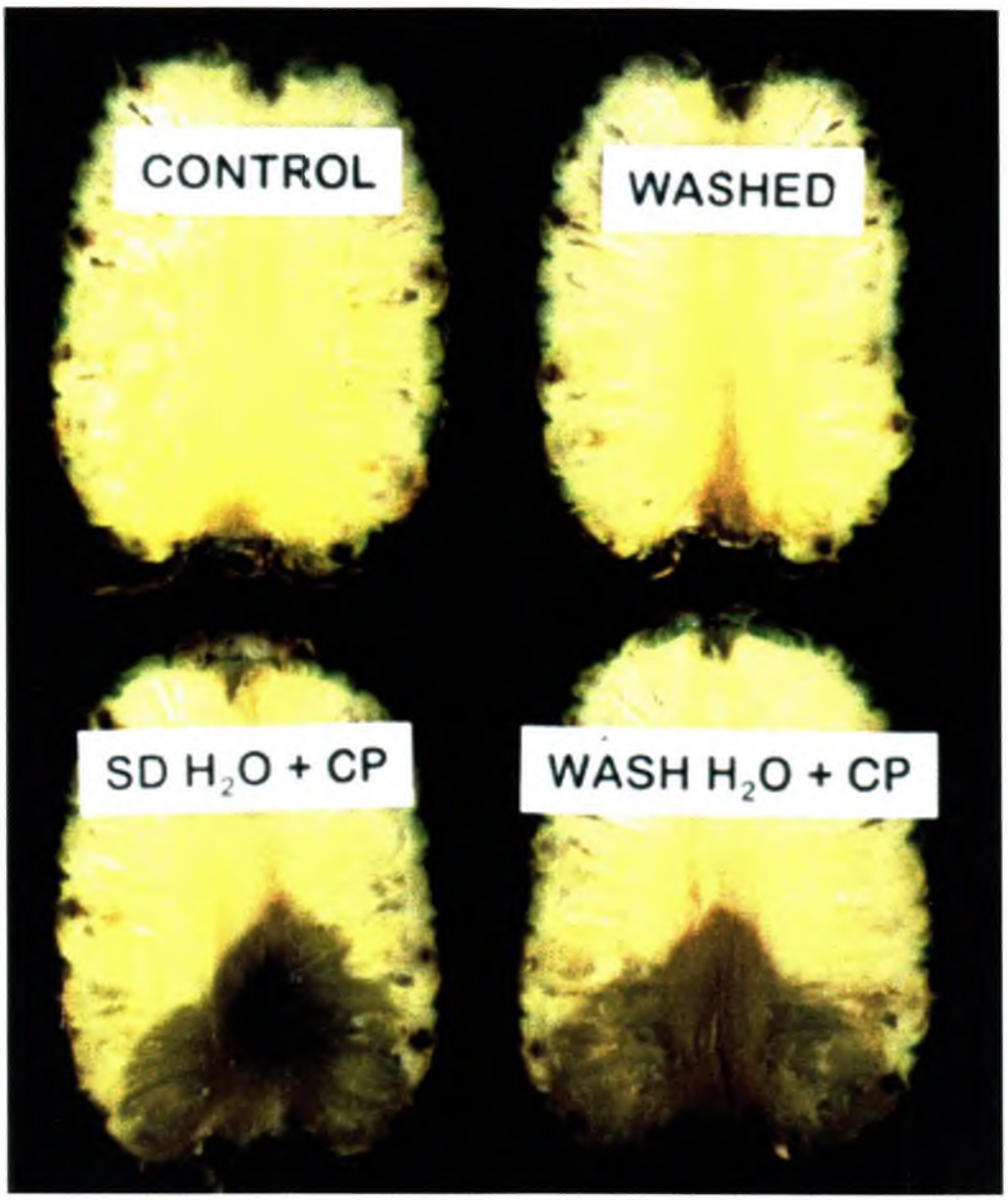

Figure 4.1. Black rot incidence and severity of pineapple fruit atomized with pineapple fruit wash water. Fruit were held at 22°C for 7 days then evaluated. Top row, left to right: Control fruit, fruit washed in sterile distilled water. Bottom row: left to right: fruit atomized with sterile distilled water or pineapple fruit wash water then *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later.

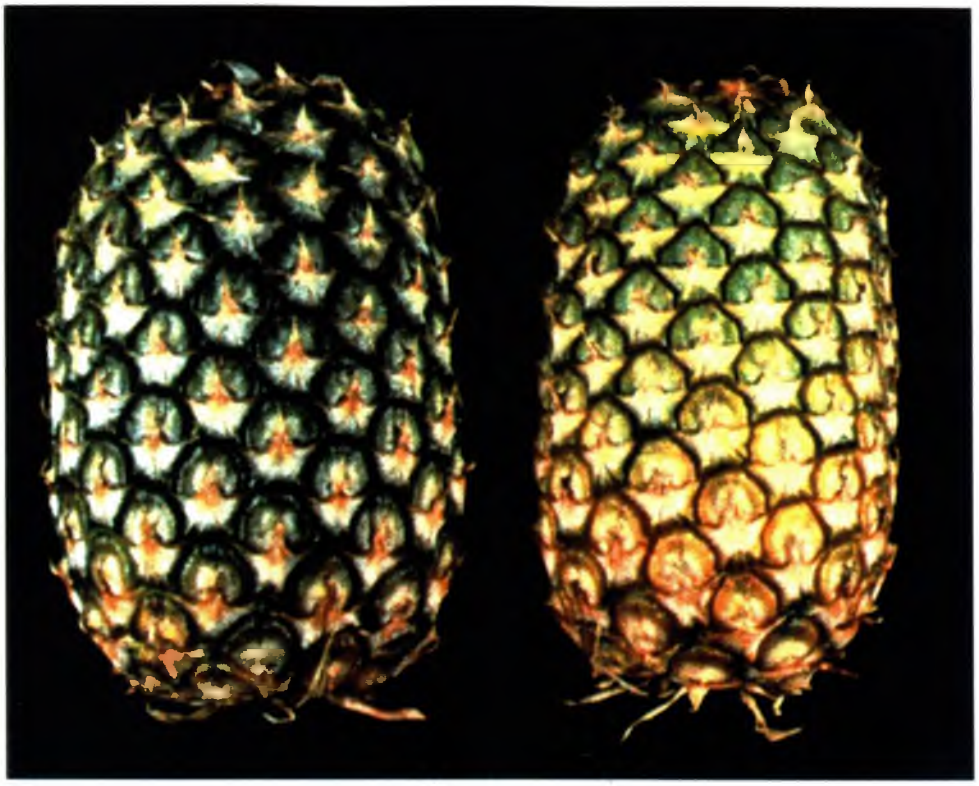

Figure 4.2. Pineapple fruit shell color: uninoculated (left) and inoculated with *Chalara paradoxa* (right).

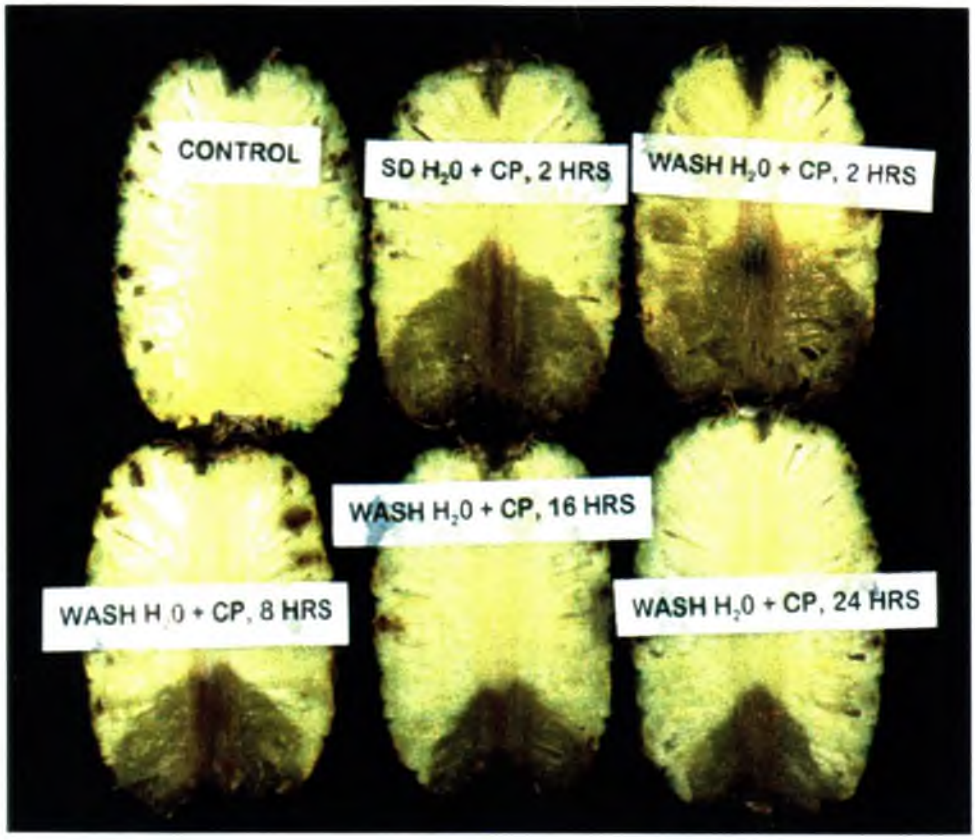

Figure 4.3. Black rot incidence and severity of pineapple fruit atomized with pineapple fruit wash water and *Chalara paradoxa* at different time intervals. Top row, left to right: Control fruit, fruit atomized with sterile distilled water or pineapple fruit wash water then *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Bottom row, left to right: fruit atomized with pineapple fruit wash water then *C. paradoxa* spore suspension 8, 16, and 24 hr later. Fruit were held at 22°C for 7 days then evaluated.

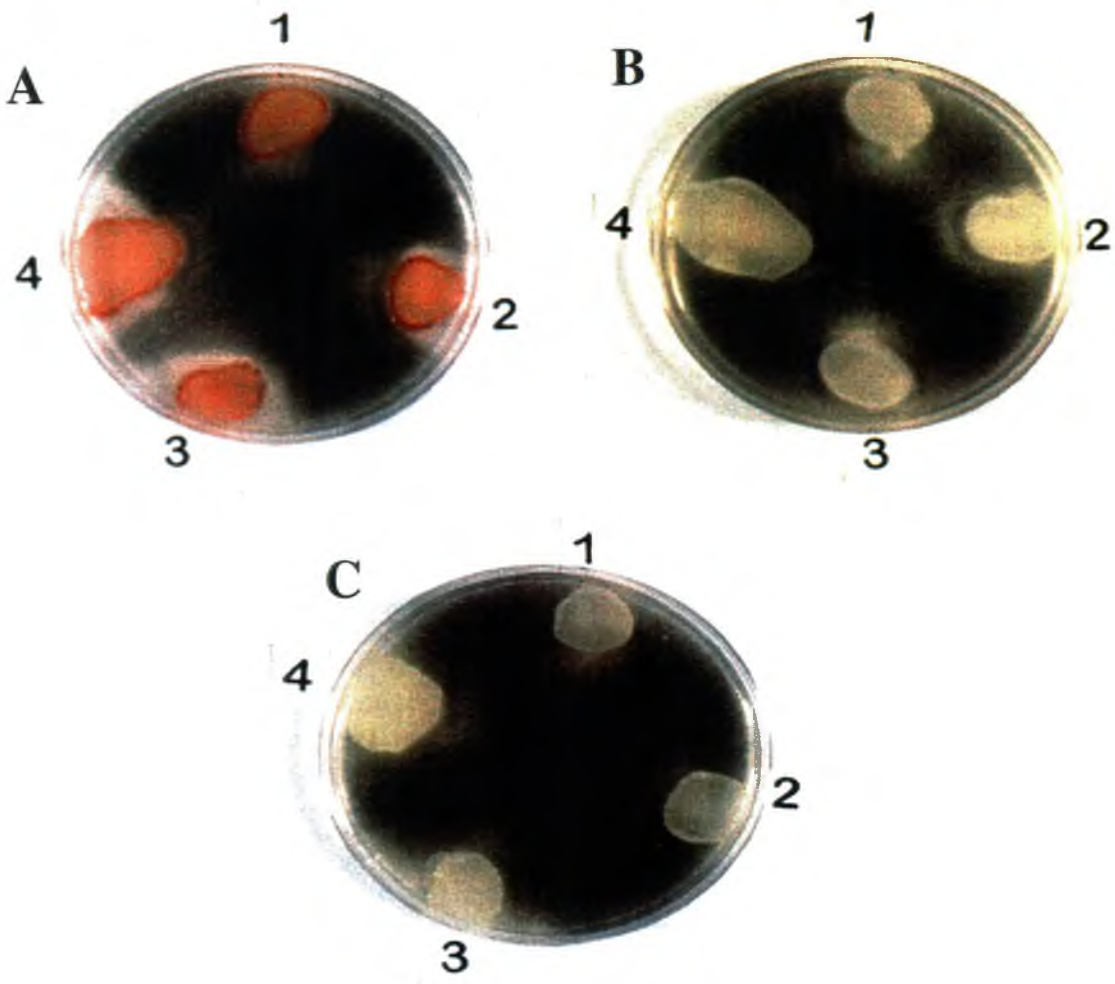

Figure 4.4. Screening of the most frequently isolated yeasts from pineapple fruit shell: (A) *Rhodoturula sp.* (Rhodoturula1), (B) *Pichia guilliermondii* (Pichia), and (C) *Cryptococcus sp.* (Cryptococcus). Yeast isolates at different inoculum concentrations (numbers 1 to 4 indicate increasing inoculum concentration; (1x): Pichia, 2.6 x 10^8 spores ml^{-1}; Rhodoturula1, 1.6 x 10^8 spores ml^{-1}; Cryptococcus, 2.3 x 10^8 spores ml^{-1}) were challenged with *Chalara paradoxa* on glucose yeast extract agar plates. Plates were held at 22°C for 7 days then evaluated for the level of inhibition.

Figure 4.5. Cut peduncle end of pineapple fruit covered with *Penicillium* mold.

4.4 Discussion

Water obtained by washing individual pineapple fruit in sterile distilled water contained beneficial organisms. The wash water was able to protect the fruit from black rot by reducing the severity fruit rot (Tables 4.1 and 4.2, Figures 4.1 and 4.3). This suggests that there is a naturally occurring epiphytic antagonist population on the surface of the pineapple fruit, long recognized by researchers in the field of biological control and described as "pathogen-suppressive soils" (Cook and Baker, 1983) or suppressive populations of nonpathogenic "plant-associated microorganisms". A possible explanation for the reduction in black rot severity is that whatever microorganism were in the wash water were able to colonize the tissues at the cut peduncle end of the fruit in advance of *C. paradoxa.* Numerous papers have reported that there are natural antagonists in the phyllosphere and rhizosphere of plants (Blakeman, 1985; Spurr and Knudsen, 1985; Roberts, 1990; Torres, 1993; McCormack et al., 1994; Urquhart et al., 1994), on fruits such as apple (Janisiewicz, 1987, 1988, 1996; Roberts, 1990; Castoria et al., 1997; Piano et al., 1997; Vinas et al., 1998), cherry (Roberts, 1990), citrus (Wilson, 1989; Wilson and Chalutz, 1989; Droby et al., 1989; McLaughlin et al.,1992; Droby et al., 1993; Mehrotra et al., 1996; El-Ghaouth, et al., 1998), grapes (McLaughlin et al.,1992; Lima et al., 1997), kiwi fruit (Lima et al., 1997), olives (Castoria et al., 1997; Chalutz and Wilson, 1990), pears (Roberts, 1990; Chand-Goyal and Spotts, 1997; Vinas et al., 1998), and strawberry (Lima et al., 1997) that can suppress disease development.

The apparent occurrence of a naturally occurring epiphytic antagonist population on the surface of the pineapple fruit led to the next step, to select potential microbial antagonists

using the procedure of Wilson et al. (1993). This strategy allows for rapid selection and testing of microorganisms on the surface of fruits and vegetables as potential antagonists against a number of postharvest pathogens. It is based on the utilization of wounds in fruit as a selective screen for potential antagonists. Wounds, inoculated with wash water obtained from fruits and vegetables, that did not develop any rot, were scraped and dislodged material diluted in sterile distilled water and plated on nutrient agar. This procedure avoids the problem of a typical approach of using primary *in vitro* screening to identify potential candidates, followed by secondary *in vivo* screening against pathogens in wounds of fruits and vegetables (Pusey, 1991). Primarily, antagonists that are antibiotic producers are identified and it may not select antagonists having other modes of action such as nutrient competition or direct parasitism (Wisniewski et al., 1991). Wilson and his co-workers used fruit that have fairly smooth skin and the surface of the pineapple fruit is quite rough with a lot of cracks in between individual fruitlets that offered numerous protected sites for microbial growth. Therefore, the typical approach was still used, although only frequently isolated filamentous fungi and yeasts were tested. Most of the filamentous fungal isolates tested were overgrown with *C. paradoxa*, however there was one isolate of *Penicillium* that looked promising (had extensive mycelial growth, and sporulated in culture) even if it was overgrown with *C. paradoxa*. However, it was not further tested because *Penicillium* mold on the cut peduncle end of the pineapple fruit is one of the problems that is seen in fresh fruit (Figure 4.5). Thus, yeast isolates were screened without knowing their identity. It later turned out that the genera of these yeasts had been reported elsewhere to be antagonists against postharvest pathogens (Roberts, 1990; Droby et al., 1993; McCormack et al., 1994; Castoria

et al., 1997; Chand-Goyal and Spotts, 1997). The *in vitro* screening of the yeast isolates showed that *C. paradoxa* was inhibited. It even appeared that the isolate Rhodoturula1 was able to inhibit sporulation of *C. paradoxa* (Figure 4.4). This was evident especially with the higher inoculum concentrations used where the yeast colonies were surrounded with white mycelial growth . Whether or not the same level of inhibition afforded by the yeasts, *in vivo*, over *C. paradoxa* is addressed in subsequent experiments.

Fruit shell color was consistently higher in fruit inoculated with *C. paradoxa* compared to the control fruit (Tables 4.1 and 4.2, Figure 4.2). Infection with a pathogen may be perceived as a wound response therefore trigger production of wound ethylene, a cascade of events that would eventually lead to degreening. The presence of ethylene could possibly affect the growth development of *C. paradoxa* as reported for the growth of *Alternaria alternata* (Kepczynska, 1994) and *Botrytis cinerea* (Kepczynska, 1993).

When fruits and vegetables are washed, they are more susceptible to decay than those that are unwashed (Wilson and Wisniewski, 1989). Uninoculated washed pineapple fruit developed black rot, while unwashed fruit did not develop black rot (Table 4.1, Figure 4.1). This has also been observed by Dole Fresh Fruit Co. (McCormack, personal communication). It is speculated that the removal of disease-suppressing antagonistic microorganisms may be a partial explanation of this phenomenon. It is also possible that the fruit was being inoculated with disease-causing microorganisms already present in the wash tanks. Another possibility was that washed fruit had more bruises than unwashed fruit, therefore providing entry points for pathogens that otherwise cannot penetrate the fruit peel. Cohen et al. (1991) reported that citrus fruit that have been submerged in water develop more sour rot than those

fruit exposed to water saturated air. This observation was not due to removal of disease-suppressing antagonistic microorganisms, rather, to more water absorbed by the peel in fruit submerged in water, predisposing the fruit to more disease.

The reduction of black rot severity (Table 4.2, Figure 4.3) in pineapple fruit with a longer interval between application of the wash water and a spore suspension of *C. paradoxa* may be due to drying out of the peduncle tissue. Roberts (1990) reported that as the interval between wounding apple fruit and inoculation with *Botrytis cinerea* increased from 0 to 72 hr, susceptibility of wounds to decay by *B. cinerea* decreased. This appears to be a wound healing response. *C. paradoxa* has been reported to require 100% relative humidity for conidial germination (Oruade-Dimaro and Ekundayo, 1992). The two-hour interval was chosen for subsequent experiments to allow for time needed in preparation of inoculum without sacrificing drying out of the peduncle tissue. Another possible explanation was that the cut peduncle end had already been colonized by whatever microorganism(s) were present in the wash water, therefore preventing infection by *C. paradoxa*

CHAPTER 5

ANTAGONIST TESTS ON PINEAPPLE FRUIT

5.1 Introduction

Confirmation of a potential microbial antagonist efficacy *in vivo* is essential to sound biological control research. A microbial antagonist may work perfectly *in vitro* but once it is tested *in vivo*, it gives inconsistent results. A good microbial antagonist against a postharvest disease pathogen must perform consistently and should be compatible with other procedures the fruit or vegetable are subjected to in the handling system. These procedures include storage at low temperatures, use of wax and or fungicides or even the manner by which the wax or fungicide is applied.

The objectives of this study were 1) Determine whether the yeast isolates that inhibited *C. paradoxa in vitro* were able to control black rot *in vivo* on pineapple fruit, 2) Determine whether the use of the yeast antagonist(s) was compatible with current industry practice of keeping pineapple fruit at low temperature (8 to 10°C) during transport, and, 3) Determine if combining yeast antagonist(s) with a low dose of Bayleton resulted in a reduction in the incidence and severity of black rot in pineapple fruit comparable to the level of control achieved with a commercial dose of Bayleton.

5.2 Materials and methods

5.2.1 Fruit handling and evaluation

Fruit handling and evaluation was similar to 4.2.1.1. Additional evaluation of fruit was done for leakage at the cut peduncle end as the percentage of total number of fruit that were leakers.

5.2.2 Cultures

Chalara paradoxa was isolated from field-harvested fruit that developed black rot. Cultures of *C. paradoxa* were maintained on potato dextrose agar (PDA) at 4°C and routinely inoculated and re-isolated from pineapple fruit to maintain pathogenicity (El-Neshawy and Wilson, 1997; Piano et al., 1997). A spore suspension of 1 x 10^6 spores ml^{-1} was prepared from 7 day old cultures grown on PDA at 27°C. This concentration was chosen based upon results of Cho et al.(1977) that showed that 1 x 10^6 spores $fruit^{-1}$ induced 100% infection. Cultures were flooded with sterile distilled water and the surface of the culture was carefully scraped with a sterile, disposable loop without disturbing the agar. The resulting suspension was filtered through two layers of Kimwipes Wipers (Kimberly-Clark Corp.). Spore concentration were determined with a hemacytometer and suspensions were used within 2 to3 hr. Tween-20 was added at a rate of 200 to 400 $\mu l\ l^{-1}$.

Yeast isolates obtained from the pineapple fruit shell were maintained on malt extract agar (MEA) slants at 4°C. A spore suspension of 1 x 10^8 spores ml^{-1} was prepared from 48 hr old cultures grown on MEA plates at 27°C. Cultures were flooded with sterile distilled water and the surface of the culture was carefully scraped with a sterile, disposable loop without disturbing the agar. The resulting suspension was vortexed for ca. 30 sec to break up any spore chains. Concentrations were determined with a hemacytometer and suspensions were used within 2 to3 hr. Tween-20 was added at a rate of 200 to 400 $\mu l\ l^{-1}$.

5.2.3 Yeast antagonists versus *C. paradoxa*

Five yeast isolates were chosen for antagonism tests against *C. paradoxa*: *Pichia guilliermondii*, 1 isolate (Pichia); *Rhodoturula* sp., 3 isolates (Rhodoturula1, Rhodoturula2,

Rhodoturula3); and *Cryptococcus* sp., 1 isolate (Cryptococcus). Pichia, Rhodoturula1, and Cryptococcus were chosen as they controlled black rot growth in initial screening on GYEA plates (Chapter 4). Rhodoturula2 and Rhodoturula3 were added since *Rhodoturula* sp., have been previously reported to control postharvest diseases (McCormack et al., 1994; Castoria et al., 1997). Fruit were inoculated with each isolate or a mixture of the five isolates, then 2 hr later challenged with *C. paradoxa*. Control fruit were atomized with sterile distilled water. Fruit were kept at room temperature in open shelves or baskets for 7 days, then evaluated for incidence and severity of black rot, shell color and leakage.

5.2.4 Low temperature storage

Fruit were inoculated with yeasts and *C. paradoxa* as described above except some were held at 8 to 10°C for 7 days then at room temperature on open shelves or baskets for a further 7 days, before evaluated for incidence and severity of black rot, shell color and leakage. Some treatments were also evaluated immediately after the low temperature storage treatment.

5.2.5 Low dose fungicide

Fruit were inoculated with yeasts and *C. paradoxa* as described above while other fruit received a low dose Bayleton (1-4(Chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanone; Bayer Corp., Michigan, MO) treatment (applied as a spray). This low dose was half the highest recommended dose on the product label. Some fruit were given a full dosage, 0.67 g l^{-1}, of Bayleton.

5.2.6 Experimental setup and data analysis

Experiments were setup in a completely randomized design with ten replications per treatment. Individual fruit served as a replicate. All experiments were repeated once. Statistical analyses were done using the general linear models procedure (Statistical Analysis Systems Institute Inc., Cary, North Carolina) with mean separation with Waller-Duncan.

5.3 Results

One of the yeast isolates, Pichia (*Pichia guilliermondii*), previously screened for antagonistic activity against *Chalara paradoxa* on GYEA plates, significantly reduced the severity of black rot in pineapple fruit compared to the control that received sterile distilled water (Table 5.1). The reduction in black rot severity did not significantly differ whether the fruit was inoculated with *C. paradoxa* 30 minutes or 1 hr after inoculation with the yeast. Black rot incidence was 100% in all fruits inoculated with *C. paradoxa* (Tables 5.1 and 5.2). Pichia has to be applied on the pineapple fruit before inoculation with *C. paradoxa* in order to reduce the severity of the black rot (Table 5.2). Testing other yeasts isolates screened on GYEA plates, Rhodoturula1 (*Rhodoturula sp.*) did not perform consistently. In one experiment, the yeast was able to reduce black rot severity compared to the control (Table 5.4, Figure 5.1) but not in another (Table 5.3). Other isolates of *Rhodoturula sp.* (Rhodoturula2 and Rhodoturula3) and an isolate of *Cryptococcus sp.* (Cryptococcus) were tested on the fruit and significantly reduced black rot severity (Tables 5.4 and 5.5), however, the results were inconsistent. Pichia therefore showed greater promise as an antagonist against *C. paradoxa.*

A yeast mixture treatment consisting of the five isolates screened (Pichia, Rhodoturula1, Rhodoturula2, Rhodoturula3, Cryptococcus) was added to a test of the individual yeast isolates. The yeast mixture significantly reduced black rot severity compared to the control (Table 5.5, Figure 5.6). The reduction in black rot severity using the yeast mixture was not significantly different from the reduction in black rot severity resulting from the use of the individual yeast isolates. Most fruit inoculated with *C. paradoxa* had 100% black rot incidence and higher shell color. Inoculation with a yeast isolate resulted in a significant reduction in the incidence of leakers, except for those inoculated with Pichia (Table 5.5).

Pineapple fruit inoculated with Pichia and *C. paradoxa,* then stored at 10°C for 1 week did not develop any black rot, and had significantly less rot compared to the control fruit and to fruit similarly inoculated but held at room temperature for 1 week (Table 5.6, Figure 5.2). When these fruit were held for another week at room temperature (Figure 5.3), rot was still significantly lower than in fruit held at room temperature for one week (Figure 5.2) and in fruit atomized with sterile distilled water and *C. paradoxa*, held at 10°C for 1 week, and another week at room temperature. The same trend was seen when a yeast mixture was tested instead of only Pichia (Table 5.7, Figures 5.4 and 5.5). Shell color was again significantly higher in fruit inoculated with *C. paradoxa* compared to the control fruit, except for those held at 10°C for one week. Leakage of fruit inoculated with the yeast mixture was significantly lower than those fruit atomized with sterile distilled water.

The yeast isolate Pichia and a yeast mixture of all the isolates screened, significantly reduced black rot in pineapple fruit compared to control fruit (Table 5.8). When both Pichia

and the yeast mixture were combined with half the dose of Bayleton used by the pineapple industry, black rot did not develop (Figure 5.8). Fruit that received a full dose of Bayleton did not develop any black rot compared to fruit receiving a half dose of Bayleton, that developed minimal disease (Figure 5.9).

Table 5.1. Black rot incidence and severity in pineapple fruit inoculated with *Pichia guilliermondii* (Pichia) and *Chalara paradoxa* (CP) at different time intervals.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	38 a
Pichia + CP, after 30 minutes	100 a	21 b
Pichia + CP, after 1 hr	100 a	27 b
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that wasdiseased.

Table 5.2. Black rot incidence and severity in pineapple fruit inoculated with *Pichia guilliermondii* (Pichia) and *Chalara paradoxa* (CP) in different orders.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	40 a
CP + Pichia, 2 hr later	100 a	42 a
Pichia + CP, 2 hr later	100a	32 b
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.3. Black rot incidence and severity and shell color in pineapple fruit inoculated with *Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1), or *Cryptococcus sp.* (Cryptococcus) and *Chalara paradoxa* (CP).[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]	Shell color [d]
Sterile distilled H_2O	0 b	0 c	4 c
Sterile distilled H_2O + CP, 2 hr later	100 a	42 a	5 b
Pichia + CP, 2 hr later	100 a	29 b	5 b
Rhodoturula1 + CP, 2 hr later	100 a	39 a	6 a
Cryptococcus + CP, 2 hr later	100 a	24 b	6 a
Analysis of Variance Pr > F	0.0001	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

[d] Fruit shell color was evaluated according to the Dole Fresh Fruit Color Standards: 0 - full green, 1 - slight color break, 2 - less than ¼ yellow, 3 - ¼ to ½ yellow, 4- ½ to ¾ yellow, 5 - ¾ to full yellow, 6 - full yellow to ¼ reddish brown, and 7 - more than ¼ reddish brown.

Table 5.4. Black rot incidence and severity in pineapple fruit inoculated with three different isolates of *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3) and *Chalara paradoxa* (CP).[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	17.5 a
Rhodoturula1 + CP, 2 hr later	100 a	12.5 b
Rhodoturula2 + CP, 2 hr later	100 a	10.5 b
Rhodoturula3 + CP, 2 hr later	100 a	13.0 b
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.5. Black rot incidence and severity, shell color, and leakage in pineapple fruit inoculated with isolates of *Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3), *Cryptococcus sp.* (Cryptococcus), a mixture of all yeast isolates and *Chalara paradoxa* (CP).[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]	Shell color [d]	Leakage [e]
Sterile distilled H_2O	0 b	0 c	4.0 e	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	37.0 a	5.4 a	100 a
Pichia + CP, 2 hr later	100 a	21.5 b	5.5 a	100 a
Rhodoturula1 + CP, 2 hr later	100 a	20.0 b	4.6 cd	0 c
Rhodoturula2 + CP, 2 hr later	100 a	24.0 b	4.3 de	60b
Rhodoturula3 + CP, 2 hr later	100 a	22.0 b	4.7 bcd	20 c
Cryptococcus + CP, 2 hr later	100 a	21.0 b	5.1 abc	10 c
Ymixt + CP, 2 hr later	100 a	18.0 b	5.2 ab	70 b
Analysis of Variance Pr > F	0.0001	0.0001	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

[d] Fruit shell color was evaluated according to the Dole Fresh Fruit Color Standards: 0 - full green, 1 - slight color break, 2 - less than ¼ yellow, 3 - ¼ to ½ yellow, 4- ½ to ¾ yellow, 5 - ¾ to full yellow, 6 - full yellow to ¼ reddish brown, and 7 - more than ¼ reddish brown.

[e] Leakage evaluation: percentage of the total number of fruit leaking.

Table 5.6. Black rot incidence and severity in pineapple fruit inoculated with *Pichia guilliermondii* (Pichia) and *Chalara paradoxa* (CP) as affected by low temperature (10°C) storage for one week.[a] Fruit were held at room temperature (22°C) and/or low temperature for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 d
Sterile distilled H_2O + CP, 2 hr later	100 a	30.5 a
Pichia + CP, 2 hr later, RT 1 wk	100 a	19.0 b
Pichia + CP, 2 hr later; 10°C 1 wk	100 a	0 d
Pichia + CP, 2 hr later,10°C 1 wk; RT 1 wk	100 a	12.0 c
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.7. Black rot incidence and severity, shell color, and leakage in pineapple fruit inoculated with a yeast mixture containing *Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus) and *Chalara paradoxa* (CP) as affected by low temperature (10°C) storage for one week.[a] Fruit were held at room temperature (22°C) for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 d
Sterile distilled H_2O + CP, 2 hr later	100 a	37.0 a
Ymixt + CP, 2 hr later, RT 1 wk	100 a	18.0 b
Ymixt + CP, 2 hr later, 10°C 1 wk	100 a	0 d
Ymixt + CP, 2 hr later 10°C 1 wk , RT 1 wk	100 a	7.5 c
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.8. Black rot incidence and severity, shell color, and leakage in pineapple fruit inoculated with an isolate of *Pichia guilliermondii* (Pichia) or a yeast antagonist mixture containing *Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus) and *Chalara paradoxa* (CP) when combined with a low dose of Bayleton.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [bf]	Severity [cf]	Shell color [df]	Leakage [ef]
Sterile distilled H_2O	0 c	0 d	2.5 b	0 b
Sterile distilled H_2O + CP, 2 hr later	100 a	19.5 a	3.6 ab	80 a
Pichia + CP, 2 hr later	100 a	8.5 b	3.7 ab	20 b
Pichia + ½ Bayleton + CP, 2 hr later	0 c	0 d	4.9 a	40 ab
Ymixt[g] + CP, 2 hr later	100 a	9.5 b	2.7 ab	20 b
Ymixt + ½ Bayleton + CP, 2 hr later	0 c	0 d	2.0 b	0 b
½ Bayleton + CP, 2 hr later	70 b	0.7 c	2.2 b	0 b
Full Bayleton + CP, 2 hr later	0 c	0 d	2.2 b	0 b
Analysis of Variance Pr > F	0.0001	0.0001	0.0356	0.0071

[a] Bayleton was applied as a spray. The full dosage of Bayleton was 0.67 g l^{-1}.

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

[d] Fruit shell color was evaluated according to the Dole Fresh Fruit Color Standards: 0 - full green, 1 - slight color break, 2 - less than ¼ yellow, 3 - ¼ to ½ yellow, 4- ½ to ¾ yellow, 5 - ¾ to full yellow, 6 - full yellow to ¼ reddish brown, and 7 - more than ¼ reddish brown.

[e] Leakage evaluation: percentage of the total number of fruit leaking.

[f] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[g] without Pichia.

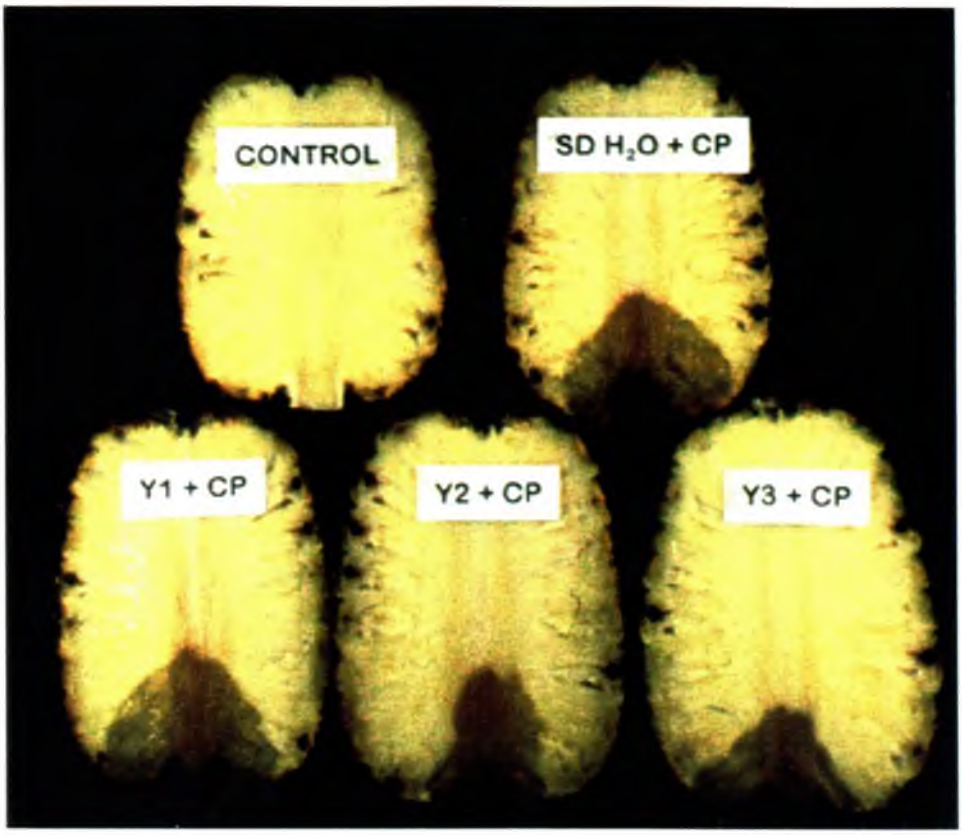

Figure 5.1. Black rot in pineapple fruit inoculated with three different isolates of *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3) and *Chalara paradoxa* (CP). Top row, left to right: Control fruit, fruit atomized with sterile distilled water then *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Bottom row, left to right: fruit atomized with Rhodoturula1 (Y1), Rhodoturula2 (Y2), or Rhodoturula3 (Y3) then *C. paradoxa* spore suspension 2 hr later. Fruit were held at 22°C for 7 days then evaluated.

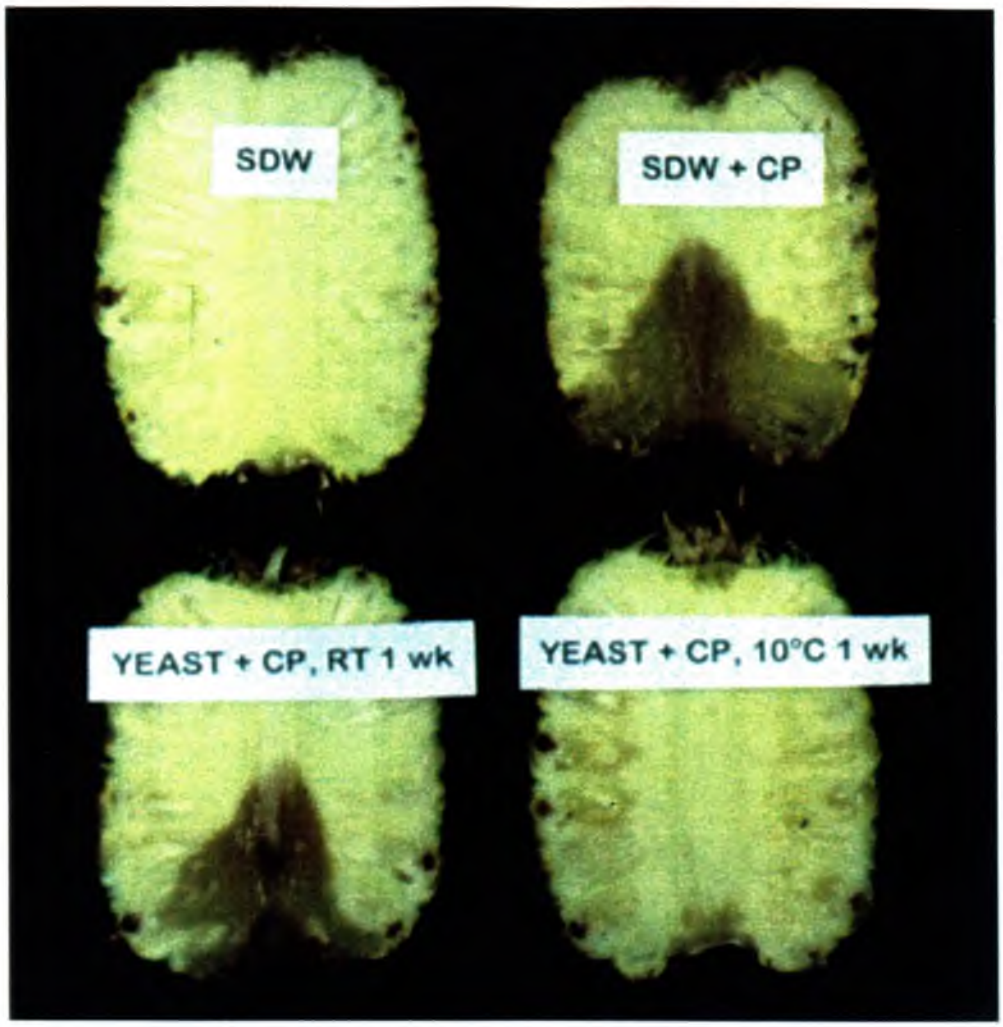

Figure 5.2. Black rot in pineapple fruit inoculated with *Pichia guilliermondii* (Pichia) and *Chalara paradoxa* (CP) as affected by low temperature (10°C) storage for one week. Top row, left to right: Control fruit, fruit atomized with sterile distilled water then *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Bottom row, left to right: fruit atomized with Pichia then *C. paradoxa* spore suspension 2 hr later and held at 22°C for one week, fruit atomized with Pichia then *C. paradoxa* spore suspension 2 hr later and held at 10°C for one week. Fruit were held at room temperature (22°C) and/or low temperature for 7 days then evaluated.

Figure 5.3. Pineapple fruit atomized with *Pichia guilliermondii* (Pichia) then *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Fruit were held at 10°C for one week, and 22°C for another week.

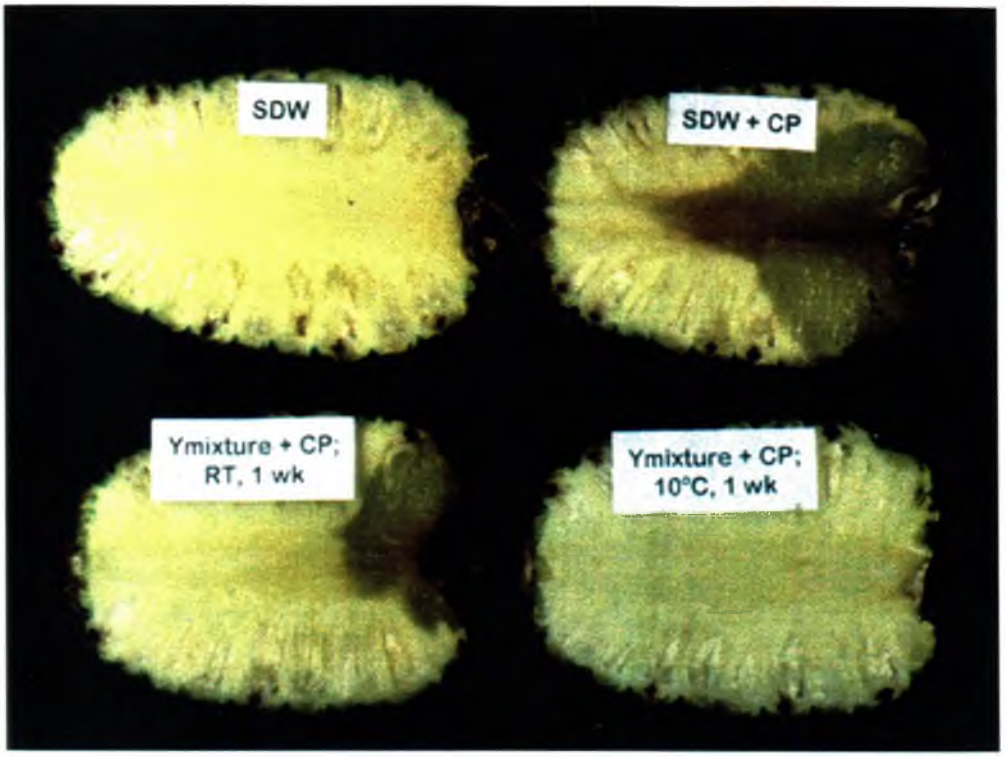

Figure 5.4. Black rot in pineapple fruit inoculated with a yeast mixture containing *Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus) and *Chalara paradoxa* (CP) as affected by low temperature (10°C) storage for one week. Top row, left to right: Control fruit, fruit atomized with sterile distilled water then *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Bottom row, left to right: fruit atomized with yeast mixture then *C. paradoxa* spore suspension 2 hr later and held at 22°C for one week, fruit atomized with yeast mixture then *C. paradoxa* spore suspension 2 hr later and held at 10°C for one week. Fruit were held at room temperature (22°C) and/or low temperature for 7 days then evaluated.

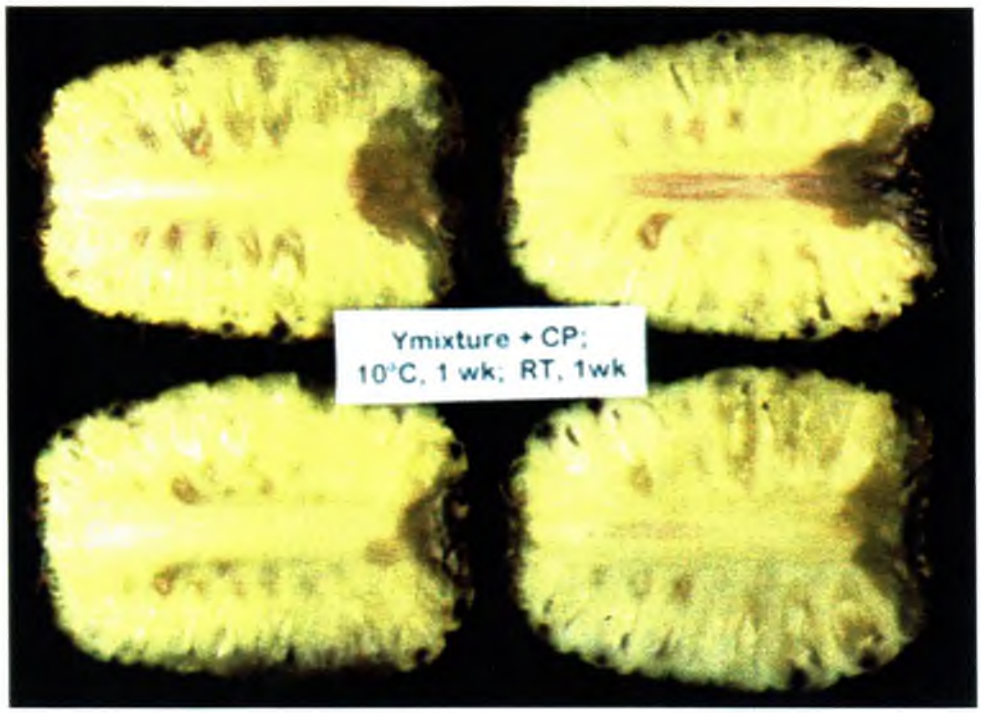

Figure 5.5. Pineapple fruit atomized with a yeast mixture then *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Fruit were held at 10°C for one week, and 22°C for another week.

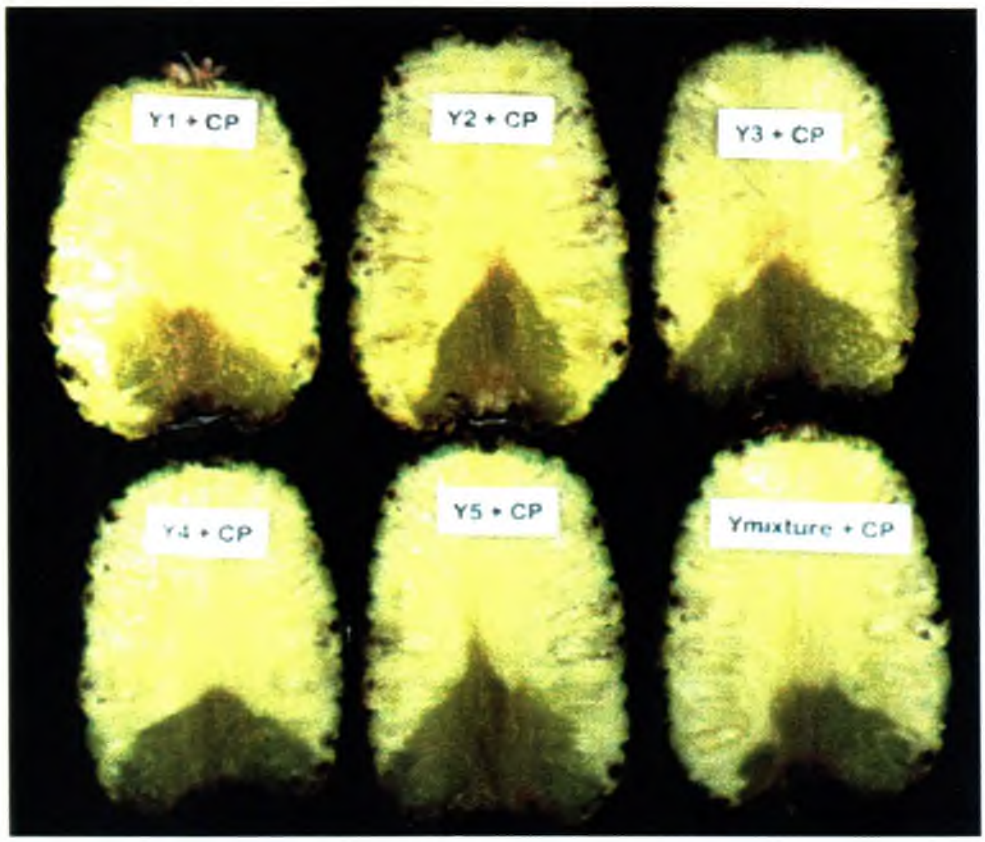

Figure 5.6. Black rot in pineapple fruit inoculated with isolates of *Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3), *Cryptococcus sp.* (Cryptococcus), a mixture of all yeast isolates and *Chalara paradoxa* (CP). Top row, left to right: fruit atomized with Pichia, Rhodoturula1, or Rhodoturula2. Bottom row, left to right: fruit atomized with Rhodoturula3, Cryptococcus, or a mixture of all five yeast isolates. All fruit were challenged with a *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Fruit were held at 22°C for 7 days then evaluated.

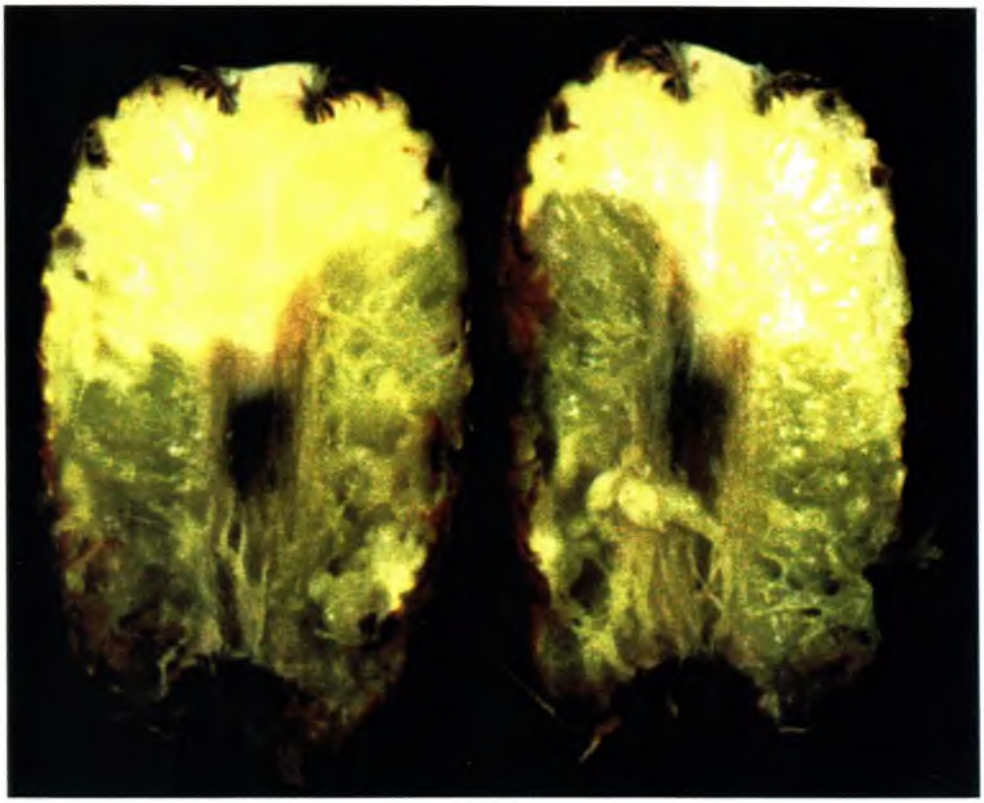

Figure 5.7. Pineapple fruit atomized with sterile distilled water then *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Fruit were held at 10°C for one week, and 22°C for another week.

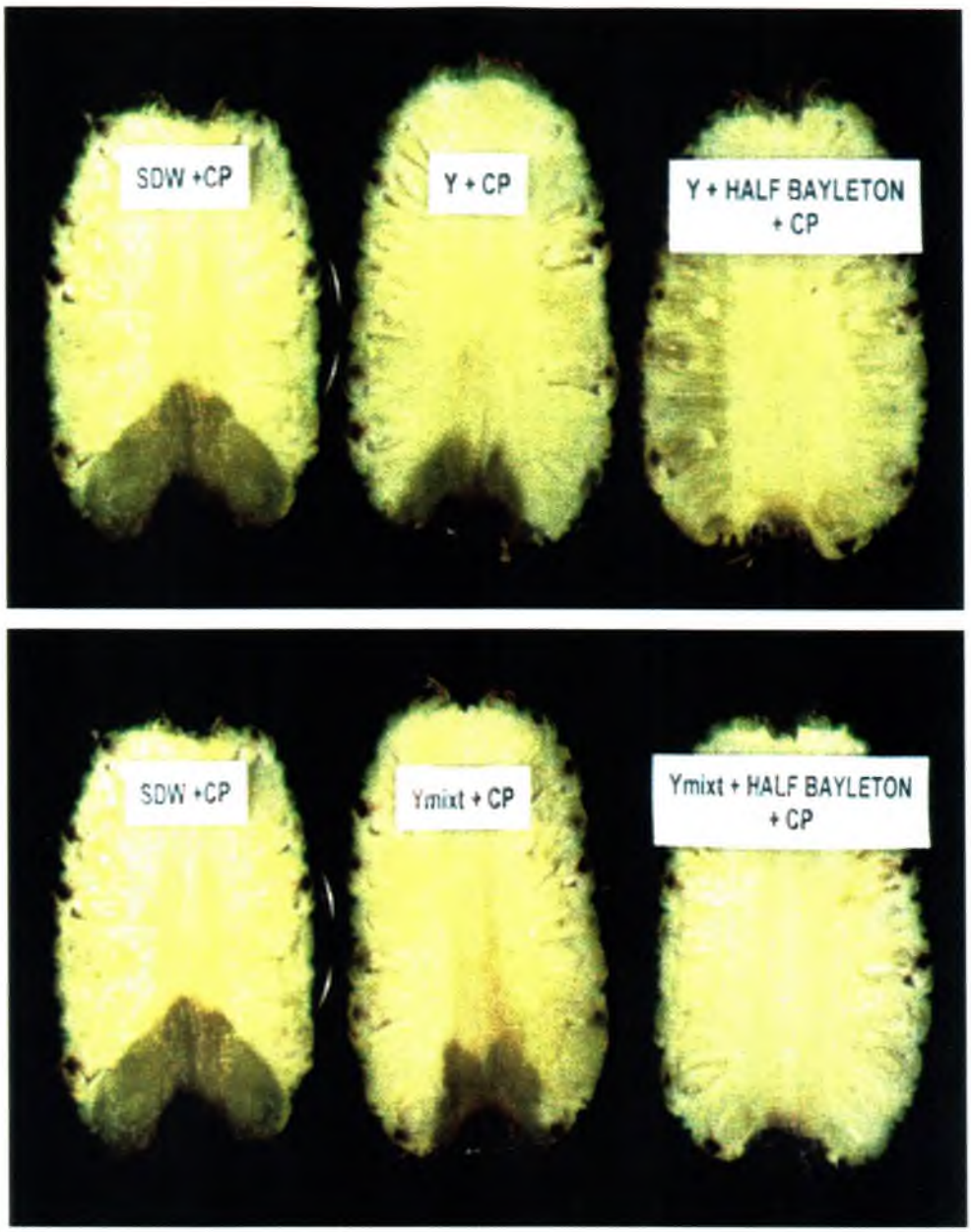

Figure 5.8. Black rot in pineapple fruit inoculated with an isolate of *Pichia guilliermondii* (Pichia) or a yeast antagonist mixture containing *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus) and *Chalara paradoxa* (CP) when combined with a low dose of Bayleton. Top row, left to right: sterile distilled water, Pichia, Pichia + half dose Bayleton. Bottom row, left to right: sterile distilled water, yeast mixture, yeast mixture + half dose Bayleton. All fruit in each treatment were challenged with a *Chalara paradoxa* spore suspension (1×10^6 spores ml^{-1}) 2 hr later. Fruit were held at 22°C for 7 days then evaluated.

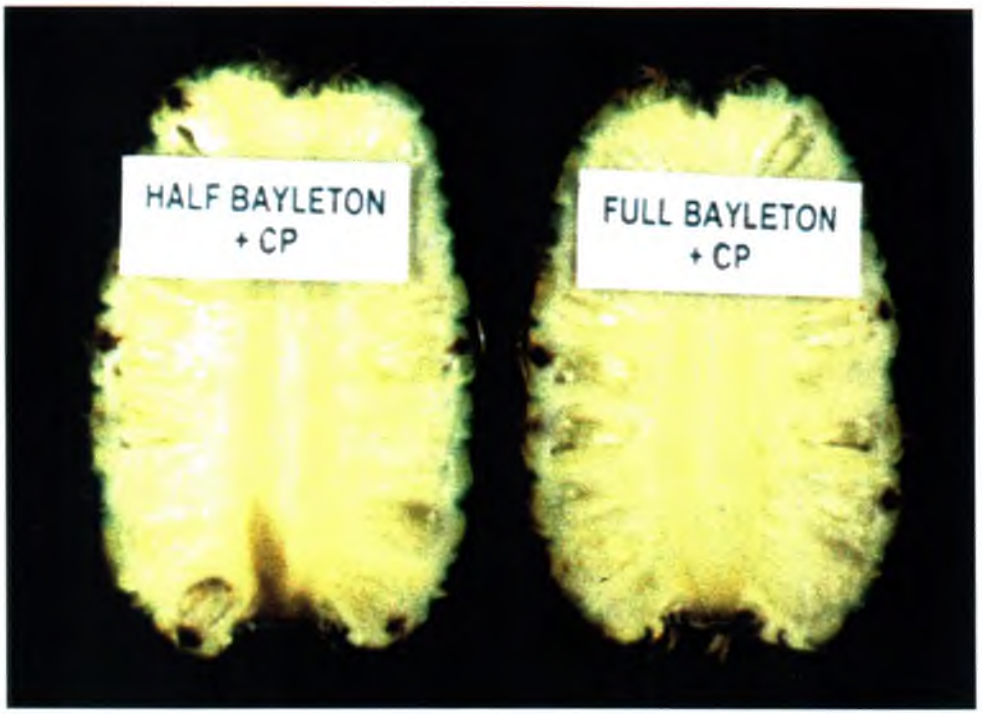

Figure 5.9. Pineapple fruit atomized with half a dose of Bayleton (left) or full dose of Bayleton (right) and challenged with a *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Fruit were held at 22°C for 7 days then evaluated.

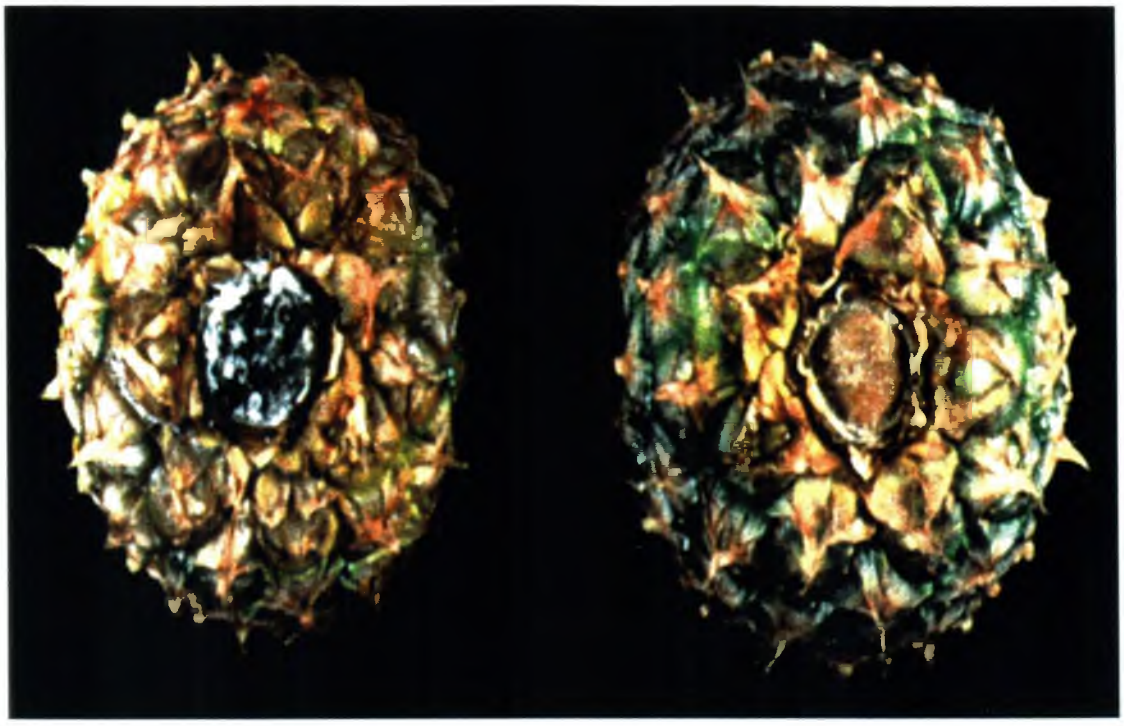

Figure 5.10. Cut peduncle end of pineapple fruit atomized with sterile distilled water (left) or a full dose of Bayleton (right) and challenged with a *Chalara paradoxa* spore suspension (1×10^6 spores ml^{-1}) 2 hr later. Fruit were held at 22°C for 7 days then evaluated.

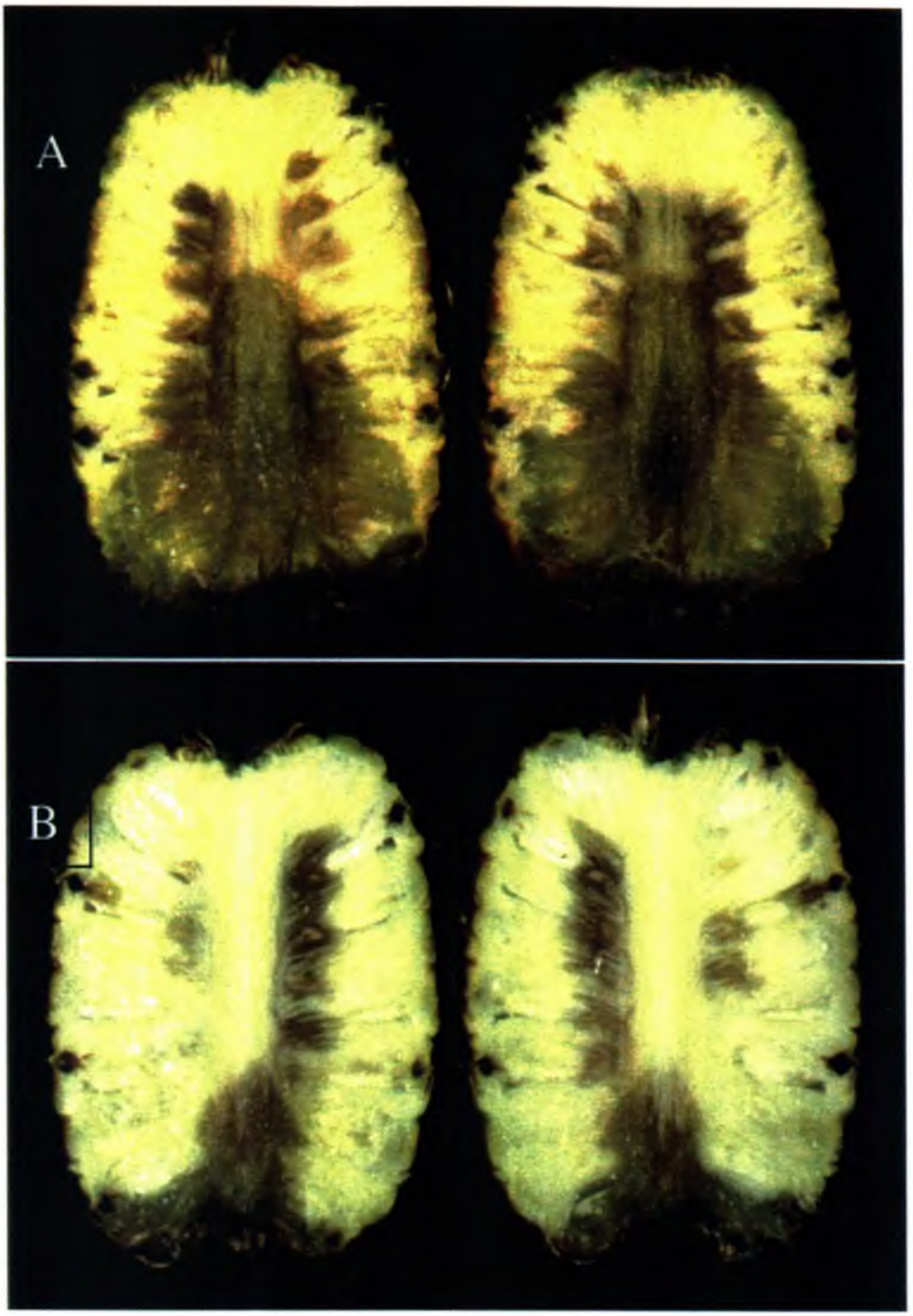

Figure 5.11. Chilling injury observed in pineapple fruit atomized with sterile distilled water (A) or *Pichia guilliermondii* (Pichia) (B) and challenged with a *Chalara paradoxa* spore suspension (1 x 10^6 spores ml^{-1}) 2 hr later. Fruit were held at 10°C for one week, and 22°C for another week.

5.4 Discussion

It was possible to reduce the severity of black rot in pineapple fruit with yeast antagonists isolated from the pineapple fruit shell. However, not all isolates performed consistently. The lack of consistency has been previously reported for biological control agents (El-Ghaouth and Wilson, 1995; Droby et al., 1998). Among the five isolates tested, however, Pichia seemed most promising, and was used in further testing with low temperature storage and in combination with a low dose of Bayleton. Efficacy of Pichia was maintained when applied prior to inoculation with *C. paradoxa*. Reduction in the severity of black rot was not seen if Pichia is applied after inoculation with *C. paradoxa*. These results suggested that in order to reduce the severity of black rot in pineapple fruit, the fruit must be colonized first by the yeast antagonist. It acted more like a protective coat on the fruit to reduce infection by *C. paradoxa*. If *C. paradoxa* had infected the pineapple fruit, the yeast antagonist did not reduce black rot severity to the same extent. Similar results have been reported for *Debaryomyces hansenii* in the control of green and blue mold and sour rot of citrus fruit (Chalutz and Wilson, 1990).

Using a yeast mixture against *C. paradoxa* did not offer an advantage over using an individual yeast antagonist alone. This result contrasted with Janisiewicz (1996) who reported that a combination of yeast isolates was far more superior at controlling blue mold in apple than individual antagonists. Nutritional profiles of these antagonists, based on utilization of 35 carbon and 33 nitrogen sources, revealed significant differences in carbon catabolism. These differences caused niche differentiation and allowed populations of each antagonist to flourish in the same wound. In the present study, the control achieved when a

yeast mixture was used against *C. paradoxa* might be due to competition for space, since the mixture was made up of the five yeast antagonists at 1 x 10^8 spores ml^{-1} each (Σ= 5 x 10^8 spores ml^{-1}). Control cannot be attributed solely to the presence of the promising antagonist, Pichia, since in one experiment, it was inadvertently left out in the preparation of the yeast mixture, and yet a reduction in black rot severity was still observed.

The isolate Pichia or the yeast mixture were able to reduce severity of black rot even when fruit were stored at low temperature, indicating that the yeasts were not adversely affected by the low storage temperatures. The results showed a significant reduction in disease than in fruit similarly treated but kept at room temperature. In terms of marketing strategies, microbial antagonists offer the seller more time to keep the fruit on the store shelves before the disease develops aggressively. Similar findings have been reported in the use of *Aureobasidium pullulans* and *Candida oleophila* on *Botrytis* and *Rhizopus* rots in strawberry (Lima et al., 1997) and *Candida sake* on *Botrytis* and *Penicillium* rots in apple (Vinas et al., 1998).

Chilling injury was seen in fruit held at 10°C for one week and further held at room temperature for another week (Figure 5.11). This was not seen in all fruit held in storage. This condition has been previously reported (Rohrbach and Paull, 1982; Paull and Rohrbach,1985).

When Pichia or the yeast mixture were combined with a half commercial dose of Bayleton, black rot did not develop (Table 5.8, Figure 5.8). This indicate that the yeast antagonists were not adversely affected by Bayleton; otherwise, black rot would have developed. The level of control achieved was equal to that of the commercial dosage of

Bayleton (Figure 5.9). This finding translates to savings realized in the market preparation of the fruit as the cost of using Bayleton would be halved. The half rate of Bayleton alone was not able to completely control black rot (Figure 5.9). The cut peduncle end of the fruit was significantly cleaner in fruit treated with Bayleton and *C. paradoxa* compared to fruit treated with sterile distilled water and *C. paradoxa* (Figure 5.10). The findings in this study supported what other researchers have reported, that yeast antagonists combined with a dilute fungicide treatment is effective in reducing postharvest rots in fruits (Droby et al., 1993, 1998; Chand-Goyal and Spotts, 1996, 1997; Piano et al., 1997). During pilot testing in a commercial packinghouse, *Pichia guilliermondii* in combination with 200 ppm thiabendazole (TBZ) (10% of the commercial rate) is able to reduce postharvest rot due to *Penicillium digitatum* in citrus fruit to a level equal to that of a commercial dosage (Droby et al., 1993). Isolates of *Cryptococcus laurentii* and *Rhodoturula glutinis* when applied separately with 15 $\mu g\ ml^{-1}$ of TBZ is able to control blue mold of pear caused by *Penicillium expansum* to the level of control achieved with 525 $\mu g\ ml^{-1}$ TBZ (Chand-Goyal and Spotts, 1996). Better control of pear diseases is achieved with a combination of either *Cryptococcus laurentii* or *Rhodoturula glutinis* and TBZ at a rate of 264 $\mu g\ ml^{-1}$ (50% of commercial rate), than at 15 $\mu g\ ml^{-1}$ (3% of commercial rate) (Chand-Goyal and Spotts, 1997). Furthermore, *Cryptococcus laurentii* with 264 $\mu g\ ml^{-1}$ TBZ is significantly more effective for blue mold control on pear, than TBZ at 528 $\mu g\ ml^{-1}$(commercial rate) alone whenever any TBZ-resistant spores were present in the inoculum. Pilot testing of Aspire (a biocontrol product containing the yeast *Candida oleophila* as the active ingredient) against postharvest decay of citrus fruit in a commercial packinghouse revealed that combined with 200 $\mu g\ ml^{-1}$ TBZ, it reduced the

incidence of green and blue molds, comparable to that of the conventional fungicide treatment (sodium O-phenyl-phenate (SOPP), TBZ, imazalil, and metataxyl) (Droby et al., 1998). Aspire is also highly efficacious against sour rot caused by *Geotrichum candidum*, a decay not controlled by the conventional treatment. Control achieved with the Aspire-TBZ combination is maintained during a 5-day period during which the fruit is shipped to Europe and subsequently held in cold storage at 5°C for 45 days. However, in shipped fruit, those treated with Aspire-TBZ had a 1% greater incidence of decay than fruit that received the conventional fungicide treatment.

CHAPTER 6

Chalara paradoxa GROWTH AS AFFECTED BY YEAST ANTAGONISTS

6.1 Introduction

The mechanism by which a microbial antagonist elicits control over a pathogen is an aid in selecting new potential antagonists, and to enhance biocontrol effectiveness by optimizing formulation and delivery systems. The information is needed for the commercial registration of the antagonist (Droby and Chalutz, 1994). Yeast antagonists have been given special attention since they effect control over a pathogen without the production of antibiotics or other toxic secondary metabolites (Smilanick, 1994). The mechanisms by which postharvest rot pathogens are controlled by yeast antagonists include: competition for space and nutrients (Chand-Goyal and Spotts, 1996; Janisiewicz, 1996; Piano et al. 1997), inhibition of spore germination and germ tube growth (Droby et al., 1989; El-Neshawy and Wilson, 1997; Piano et al., 1997), production of extracellular hydrolases (Wisniewski et al., 1991), maintenance of normal metabolism at high osmotic potentials (Wisniewski et al., 1995), and the induction of resistance responses in the host tissue (Arras, 1996; El-Ghaouth et al., 1998).

One of the first things that is considered when evaluating a biocontrol agent is the growth of the pathogen that is being controlled. Therefore, the objective of this study was to determine whether growth of *Chalara paradoxa*, in terms of spore germination and germ tube length, is inhibited in the presence of yeast antagonists.

6.2 Materials and methods

6.2.1 Cultures

The cultures used and the manner in which they were grown and stored were similar to those described in 5.2.2. Yeast isolates tested were: *Pichia guilliermondii*, 1 isolate (Pichia); *Rhodoturula* sp., 3 isolates (Rhodoturula1, Rhodoturula2, Rhodoturula3); and *Cryptococcus* sp., 1 isolate (Cryptococcus).

6.2.2 Spore germination tests

The effect of yeast on the germination of *C. paradoxa* spores was done in 96-well plates (Falcon 3072, Microtest III Tissue Culture Plate, sterile, flat bottom with low evaporation lid, Becton Dickinson Labware, Becton Dickinson & Co., Lincoln Park, NJ). *C. paradoxa* spore suspension was prepared using sterile pineapple juice filtrate at a concentration of 1×10^6 spores ml^{-1}. The pineapple juice filtrate was prepared by extracting the juice from field-ripened fruit (Figure 3.1, shell color 6) using a juice press. The juice was filtered through 6 layers of cheesecloth and then an equal amount of distilled water was added. The juice filtrate was autoclaved at 121°C for 15 minutes. This was then filtered through sterile Whatman No. 1 filter paper and again filter sterilized with a Cameo 25AS acetate syringe filter, 0.22 micron (Micron Separations, Inc., Westboro, MA). Yeast suspensions were prepared using sterile distilled water and were used at a concentration of 1×10^8 spores ml^{-1}. A yeast mixture containing all five yeast isolates was prepared by mixing together equal amounts of each yeast isolate at a concentration of 1×10^8 spores ml^{-1} each. A 100 µl aliquot of each of the suspensions were added to a well. After 24 hr incubation at

room temperature (22°C), spore germination was evaluated and germ tube length measured microscopically using an ocular micrometer.

6.2.3 Hyphal growth of *C. paradoxa*

6.2.3.1 Solid medium

On glucose yeast extract agar (GYEA: 3 g glucose, 1 g yeast extract, 20 g agar 1 L distilled water) plates, a 100 µl aliquot of yeast antagonist suspension (prepared with sterile distilled water at a concentration of 1 x 10^8 spores ml^{-1}) or yeast mixture (prepared as in 6.2.2) was spread and a 20 µl aliquot of *C. paradoxa* spore suspension (prepared with sterile distilled water at a concentration of 1 x 10^6 spores ml^{-1}) added to the middle of the plate. Plates were incubated at room temperature for 7 days, and the diameter of *C. paradoxa* growth measured.

6.2.3.2 Liquid medium

Growth of *C. paradoxa* in sterile pineapple juice filtrate amended with yeast antagonists was evaluated. Ten ml of sterile pineapple juice filtrate in a 100 mm petri plate was amended with 100 µl of a yeast antagonist suspension (prepared with sterile distilled water at a concentration of 1 x 10^8 spores ml^{-1}) or yeast mixture (prepared as in 6.2.2). Two 4 mm-discs of *C. paradoxa* grown on PDA for 7 days or a 200 µl *C. paradoxa* spore suspension (prepared with sterile distilled water at a concentration of 1 x 10^6 spores ml^{-1}) was added to the plate. Plates were incubated at room temperature for 7 days. Dry weight of *C. paradoxa*, after growth in the presence or absence of the yeast antagonist, were determined by collecting the culture on Whatman No. 1 filter paper and dried at 80°C for 12 hr.

6.2.4 Experimental setup and data analysis

Experiments were setup in a completely randomized design with ten replications per treatment. In the spore germination experiments, individual wells in the 96-well plate served as a replicate. Thirty spores per well were evaluated under the microscope. In the hyphal growth experiments, individual plates served as a replicate. All experiments were repeated once. Statistical analysis was done using the general linear models procedure (Statistical Analysis Systems Institute Inc., Cary, North Carolina) and mean separation with Waller-Duncan.

6.3 Results

Percent spore germination of *C. paradoxa* was greatly inhibited in the presence of yeast antagonists compared to the control (Table 6.1, Figure 6.1). The greatest inhibition was seen with the isolate Pichia (*Pichia guilliermondii*) and the least with the isolate Cryptococcus (*Cryptococcus sp.*). A similar result was obtained in germ tube length.

Growth of *C. paradoxa* on GYEA plates spread with a yeast antagonist was significantly inhibited compared to the controls (Table 6.2, Figure 6.3). Growth of *C. paradoxa* in the control plates, which had been spread with sterile distilled water, reached the edge of the plate. The greatest inhibition in growth was seen with the isolate Pichia and the yeast mixture, while the least was seen with the isolate Cryptococcus.

Growth of *C. paradoxa* on pineapple juice filtrate amended with a yeast antagonist was significantly inhibited compared to the controls (Table 6.3, Figure 6.2). *C. paradoxa* in control plates had the greatest fungal weight when collected on filter paper and dried in an

80°C oven for 12 hr. The least fungal weight was obtained on plates amended with the Pichia isolate and the yeast mixture.

Table 6.1. Spore germination and germ tube length of *Chalara paradoxa* with and without yeast antagonists.[a] Plates were held at 22°C for 24 hr then evaluated.

Treatment [b]	% Spore germination [c]	Germ tube length (mm)[c]
Sterile distilled H_2O + CP	19.64 a	0.09 b
Pichia + CP	0.34 e	0 e
Rhodoturula1 + CP	5.16 bcd	0.04 cd
Rhodoturula2 + CP	7.22 bc	0.06 bc
Rhodoturula3 + CP	1.36 de	0.02 de
Cryptococcus + CP	8.96 b	0.13 a
Ymixt + CP	3.08 cde	0.03 cde
Analysis of Variance Pr > F	0.0001	0.0001

[a] A 100 µl aliquot of a spore suspension (10^6 spores ml^{-1}) of *C. paradoxa* prepared in sterile pineapple juice filtrate was added to individual wells of a 96-well plate. A 100 µl aliquot of yeast suspension (10^8 spores ml^{-1}) or yeast mixture (10^8 spores ml^{-1} of each of five yeast isolates) was added to each well.

[b] The yeast isolates tested were *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus). The yeast mixture contained all five yeast isolates.

[c] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

Table 6.2. Radial growth of *Chalara paradoxa* (CP) on glucose yeast extract agar plates with and without yeast antagonists.[a] Plates were held at 22°C for 7 days then evaluated.

Treatment[b]	CP growth diameter (mm)[c]
Sterile distilled H_2O + CP	90.0 a
Pichia + CP	2.0 e
Rhodoturula1 + CP	7.0 d
Rhodoturula2 + CP	10.0 c
Rhodoturula3 + CP	10.0 c
Cryptococcus + CP	27.0 b
Ymixt + CP	2.5 e
Analysis of Variance Pr > F	0.0001

[a] A 100 µl aliquot of yeast suspension (10^8 spores ml^{-1}) or yeast mixture (10^8 spores ml^{-1} of each of five yeast isolates) was spread on the glucose yeast extract agar plate and a 20 µl aliquot of *C. paradoxa* spore suspension (10^6 spores ml^{-1}) was added to the middle of the plate.

[b] The yeast isolates tested were *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus). The yeast mixture contained all five yeast isolates.

[c] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

Table 6.3. Hyphal growth of *Chalara paradoxa* (CP) on sterile pineapple juice filtrate in plates with and without yeast antagonists.[a] Plates were held at 22°C for 7 days then evaluated.

Treatment [b]	CP growth weight (gm)[c]	
	Disc	Aliquot
Sterile distilled H_2O + CP	0.336 a	0.303 a
Pichia + CP	0.119 b	0.186 c
Rhodoturula1 + CP	0.274 a	0.250 b
Rhodoturula2 + CP	0.292 a	0.244 b
Rhodoturula3 + CP	0.304 a	0.259 ab
Cryptococcus + CP	0.287 a	0.250 b
Ymixt + CP	0.156 b	0.152 c
Analysis of Variance Pr > F	0.0008	0.0001

[a] Two 4- mm discs of *C. paradoxa* taken from a 7 day old culture grown at 27°C or a 200 µl aliquot of *C. paradoxa* spore suspension (10^6 spores ml^{-1}) was added to 10 ml sterile pineapple juice filtrate in plates amended with a 100 µl aliquot of yeast suspension (10^8 spores ml^{-1}) or yeast mixture (10^8 spores ml^{-1} of each of five yeast isolates) .

[b] The yeast isolates tested were *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus). The yeast mixture contained all five yeast isolates.

[c] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

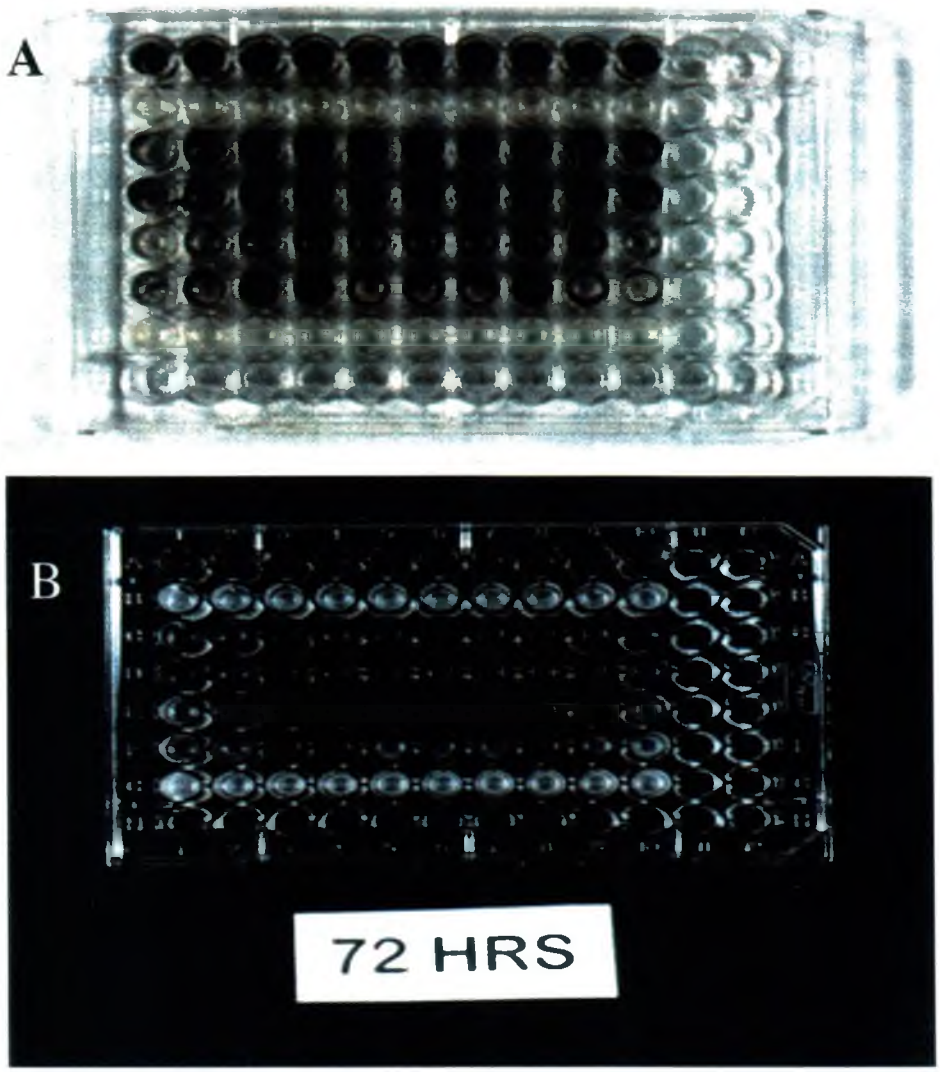

Figure 6.1. 96-well plate used for the study of spore germination and germ tube length of *Chalara paradoxa* with and without yeast antagonists. Each well contained a 100 µl aliquot of (rows top to bottom): sterile distilled water, a yeast suspension (10^8 spores ml^{-1}) of *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), *Cryptococcus sp.* (Cryptococcus), or a yeast mixture containing all five isolates or yeast mixture (10^8 spores ml^{-1} of each of five yeast isolates) and a 100 µl aliquot of a spore suspension (10^6 spores ml^{-1}) of *C. paradoxa* prepared in sterile pineapple juice filtrate. Plates were held at 22°C for 24 hr then evaluated. This plate was held for 72 hr. Plate A showing sporulation in wells containing sterile distilled water, *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus). Plate B showing high turbidity in wells containing *Pichia guilliermondii* (Pichia) and the yeast mixture.

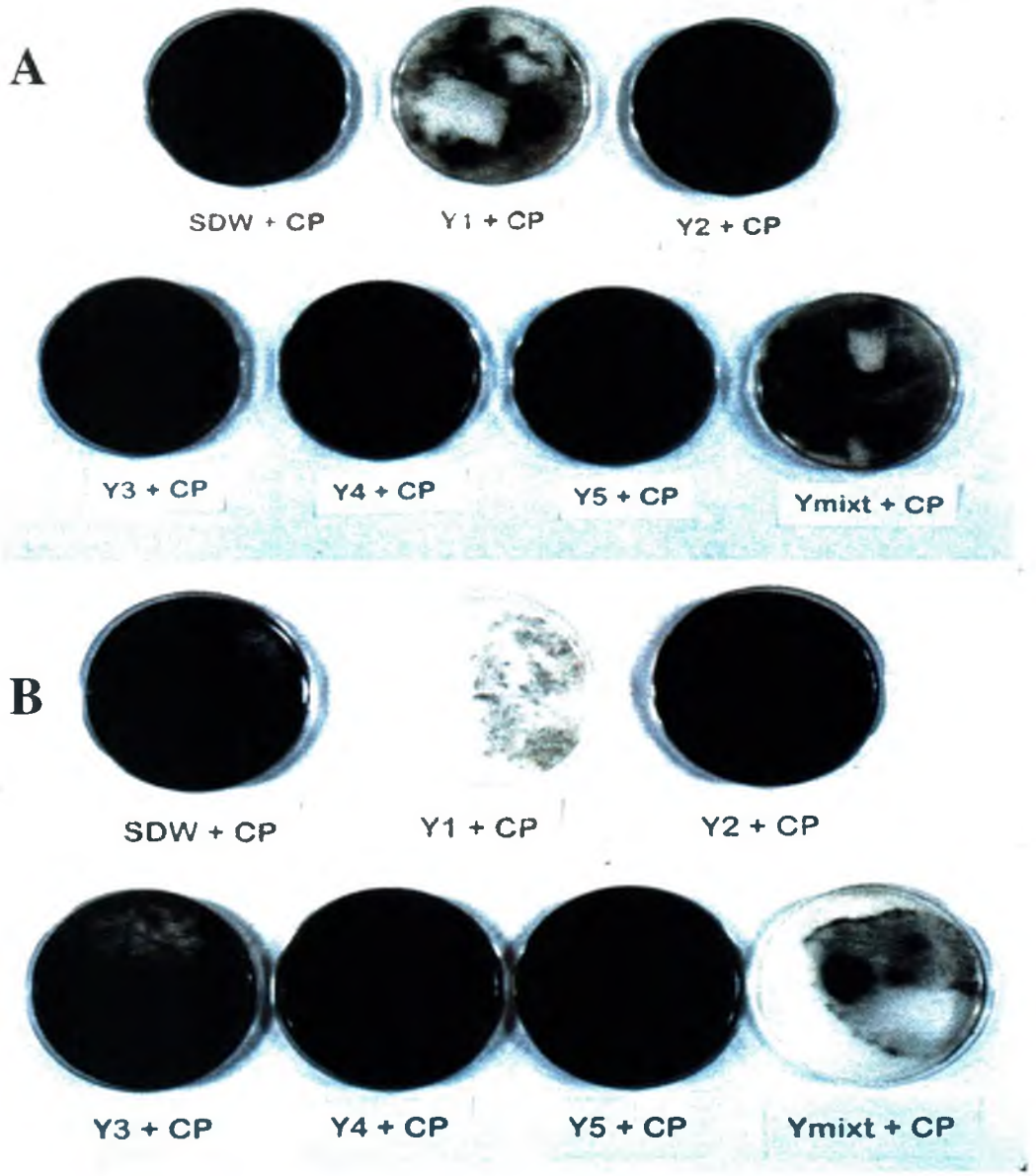

Figure 6.2. Hyphal growth of *Chalara paradoxa* on sterile pineapple juice filtrate in plates with and without yeast antagonists. Ten ml sterile pineapple juice filtrate in petri plates were amended with 100 µl aliquot of sterile distilled water, yeast suspension (10^8 spores ml^{-1}) of *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1) (A and B, top row, left to right) *Rhodoturula sp.*(Rhodoturula2, Rhodoturula3), *Cryptococcus sp.* (Cryptococcus), or a yeast mixture (10^8 spores ml^{-1} of each of five yeast isolates) (A and B, bottom row, left to right). Two 4- mm discs of *C. paradoxa* taken from a 7 day old culture grown at 27°C (A) or a 200 µl aliquot of *C. paradoxa* spore suspension (10^6 spores ml^{-1}) (B) was added. Plates were held at 22°C for 7 days then evaluated.

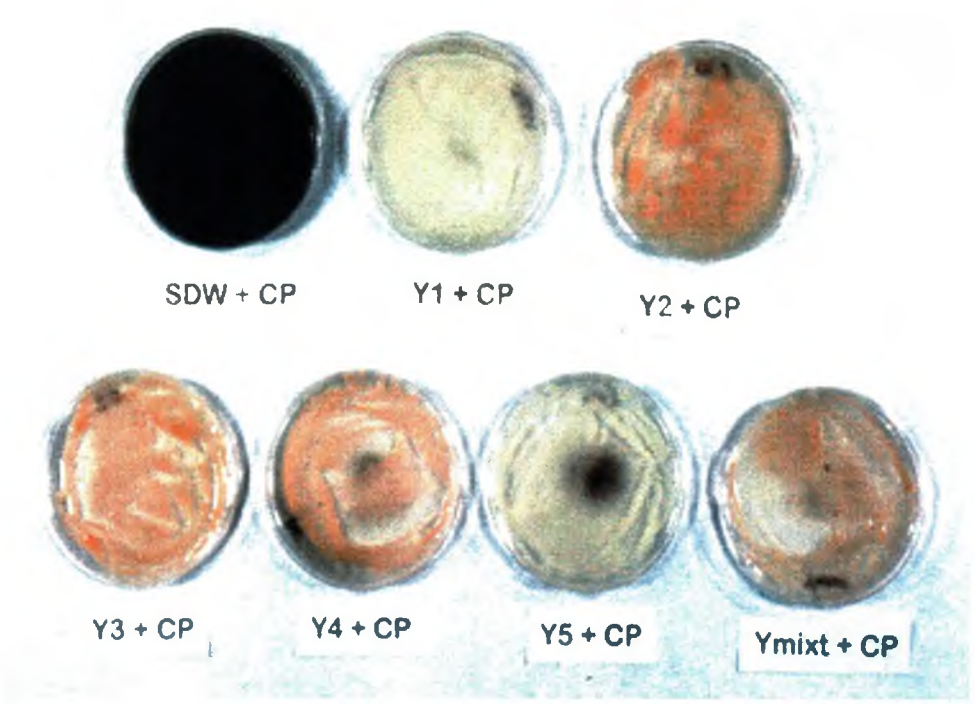

Figure 6.3. Radial growth of *Chalara paradoxa* on glucose yeast extract agar plates with and without yeast antagonists. Top row, left to right: sterile distilled water, *Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1). Bottom row, left to right: *Rhodoturula sp.*(Rhodoturula2, Rhodoturula3), *Cryptococcus sp.* (Cryptococcus), and yeast mixture containing all five isolates. Plates were held at 22°C for 7 days then evaluated.

6.4 Discussion

Percent spore germination and germ tube length of *C. paradoxa* was greatly inhibited in the presence of yeast antagonists, alone or as a mixture (Table 6.1). This provided an explanation for the earlier results (Chapter 5) where the yeast isolates significantly reduced black rot in pineapple fruit (i.e., Table 5.6). However, when the 96-well plates were incubated longer (2 weeks), it was observed that *C. paradoxa* did eventually sporulate in the wells with sterile distilled water, Rhodoturula1, Rhodoturula2, Rhodoturula3, and Cryptococcus (Figure 6.1). In the Pichia or the yeast mixture wells, a very turbid solution was observed after two weeks of incubation. Several papers have reported that yeast antagonists inhibited pathogen spore germination and germ tube growth (Droby et al., 1989; El-Neshawy and Wilson, 1997; Piano et al., 1997). These published results were interpreted as nutrient competition being the mode of action.

Growth of *C. paradoxa* on solid (GYEA) or liquid (pineapple juice filtrate) media was greatly reduced in the presence of yeast antagonists, alone or in a mixture (Tables 6.2 and 6.3, Figures 6.2 and 6.3). The greatest growth inhibition was seen with the Pichia isolate and the yeast mixture. Similar results have been reported by Droby et al. (1989) and Piano et al. (1997) for hyphal growth inhibition in the presence of a yeast antagonist. The result suggested that the mode of action of the Pichia isolate was by competition for space and nutrients. The yeast Pichia was apparently able to multiply rapidly compared to the other yeast antagonists, resulting in the turbid solution observed in the wells (Figure 6.1). The mode of action for the yeast mixture was possibly competition for space.

CHAPTER 7

CONCLUSION

Yeasts were the majority of the microbes on the pineapple fruit shell followed by filamentous fungi. Generally, there was no relationship between black rot incidence or severity and total microbial, filamentous fungi or yeast counts. There was a negative correlation between black rot incidence in wet fruit and filamentous fungal count in wet fruit, suggesting that some of the filamentous fungi on the fruit were washed away, predisposing the fruit to black rot. There were indirect temperature effects on microbial growth, resulting in black rot incidence and severity being correlated to temperatures one, up to three months before harvest. Rainfall apparently did not play a major role in black rot incidence and severity though total microbial counts were correlated to rainfall in the month of harvest.

There was a naturally occurring epiphytic antagonist population on the pineapple fruit as evidenced by the reduction of black rot severity in fruit treated with pineapple fruit wash water and *Chalara paradoxa*. Screening of potential antagonists, *in vitro*, showed that filamentous fungi on the pineapple fruit were easily overgrown by *C. paradoxa* while the most frequently isolated yeasts were able to control *C. paradoxa*.

The yeast isolates *(Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus)) evaluated in this study for antagonism against *C. paradoxa* gave *in vivo* control *C. paradoxa*. However,except for the Pichia isolate, most of the isolates did not give consistent results. Pichia needed to be applied to the pineapple fruit prior to application of *C. paradoxa* spores to reduce black rot severity. A yeast mixture containing all five yeast isolates that were individually tested, was

able to reduce black rot severity. The use of Pichia or the yeast mixture was compatible with current industry practice of holding fruit at a low temperature (10°C) and the use of Bayleton. Combining the Pichia isolate or the yeast mixture with a half dose of Bayleton resulted in complete control of black rot comparable to control achieved with a commercial dose of Bayleton.

The Pichia isolate and the yeast mixture containing all five isolates tested reduced spore germination, germ tube length, and dry matter weight of *C. paradoxa*. The mode of action by Pichia appeared to be competition for space and nutrients. Pichia was able to multipl rapidly as evidenced by the turbidity of the solution in the wells. The mode of action for the yeast mixture appeared to be competition for space.

The yeast isolate Pichia was the most promising of all the isolates evaluated. Further work on the population dynamics of this yeast isolate is needed.

APPENDIX

Table 4.1A. Black rot incidence and severity of pineapple fruit inoculated by atomizing with pineapple fruit wash water and *Chalara paradoxa*.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Unwashed control	10 b	3 b
Washed control	0 b	0 b
Sterile distilled H_2O + CP, 2 hr later	100 a	32 a
Wash H_2O + CP, 2 hr later	100 a	26 b
Analysis of variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 4.2A. Black rot incidence and severity of pineapple fruit inoculated by atomizing with pineapple fruit wash water and *Chalara paradoxa* at different time intervals.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	21 a
Wash H_2O + CP, 2 hr later	100 a	19 a
Wash H_2O + CP, 8 hr later	100 a	16 b
Wash H_2O + CP, 16 hr later	100 a	15 b
Wash H_2O + CP, 24 hr later	100 a	14 b
Analysis of variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 20).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 4.4A. Screening of most frequently isolated yeast isolates *Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1), and *Cryptococcus sp.* (Cryptococcus) from pineapple fruit shell, at different inoculum concentrations, against *Chalara paradoxa* (CP) on glucose yeast extract agar (GYEA) plates.[a] Plates were held at 22°C for 7 days then evaluated.

Yeast antagonist	Inoculum concentration[b]/ Level of control[cd]			
	1/4x	1/2x	1x	2x
WFO	1 c	1 c	1 c	1 c
O1d	1.3 b	1.3 b	1 c	1 c
SWC	1.9 a	1.5 b	1.5 b	1.3 b
Analysis of variance Pr > F		0.0001		

[a] A disc of *C. paradoxa* was placed in the center of the plate and aliquots of different concentrations of the yeast isolate were placed at the plate margins.

[b] Inoculum concentrations (1x) used were as follows: Pichia, 2.6 x 10^8 spores ml^{-1}; Rhodoturula1, 1.6 x 10^8 spores ml^{-1}; Cryptococcus, 2.3 x 10^8 spores ml^{-1}.

[c] Evaluated using the following scale: 1 = complete inhibition, 2 = partial inhibition, and 3 = no inhibition, overgrown with CP.

[d] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column and row followed by the same letter were not significantly different (n = 10).

Table 5.1A. Black rot incidence and severity in pineapple fruit inoculated with *Pichia guilliermondii* (Pichia) and *Chalara paradoxa* (CP) at different time intervals.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	26.0 a
Pichia + CP, after 30 minutes	100 a	17.5 b
Pichia + CP, after 1 hr	100 a	18.0 b
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.2A. Black rot incidence and severity in pineapple fruit inoculated with *Pichia guilliermondii* (Pichia) and *Chalara paradoxa* (CP) in different orders.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	24 a
CP + Pichia, 2 hr later	100 a	24 a
Pichia + CP, 2 hr later	100a	18 b
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.3A. Black rot incidence and severity in pineapple fruit inoculated with *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1), or *Cryptococcus sp.* (Cryptococcus) and *Chalara paradoxa* (CP).[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 d
Sterile distilled H_2O + CP, 2 hr later	100 a	27.0 a
Pichia + CP, 2 hr later	100 a	15.0 c
Rhodoturula1 + CP, 2 hr later	100 a	24.0 ab
Cryptococcus + CP, 2 hr later	100 a	18.5 bc
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.4A. Black rot incidence and severity in pineapple fruit inoculated with three different isolates of *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3) and *Chalara paradoxa* (CP).[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	22.0 ab
Rhodoturula1 + CP, 2 hr later	100 a	19.5 b
Rhodoturula2 + CP, 2 hr later	100 a	25.5 a
Rhodoturula3 + CP, 2 hr later	100 a	20.0 ab
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.5A. Black rot incidence and severity in pineapple fruit inoculated with isolates of *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), *Cryptococcus sp.* (Cryptococcus), a mixture of all yeast isolates and *Chalara paradoxa* (CP).[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	26.5 a
Pichia + CP, 2 hr later	100 a	16.5 b
Rhodoturula1 + CP, 2 hr later	100 a	16.5 b
Rhodoturula2 + CP, 2 hr later	100 a	17.5 b
Rhodoturula3 + CP, 2 hr later	100 a	17.0 b
Cryptococcus + CP, 2 hr later	100 a	14.5 b
Ymixt + CP, 2 hr later	100 a	16.0 b
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.6A. Black rot incidence and severity in pineapple fruit inoculated with *Pichia guilliermondii* (Pichia) and *Chalara paradoxa* (CP) as affected by low temperature (10°C) storage for one week.[a] Fruit were held at room temperature (22°C) and/or low temperature for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 c
Sterile distilled H_2O + CP, 2 hr later	100 a	17.5 a
Pichia + CP, 2 hr later, RT 1 wk	100 a	14.5 ab
Pichia + CP, 2 hr later; 10°C 1 wk	100 a	0 c
Pichia + CP, 2 hr later,10°C 1 wk; RT 1 wk	100 a	11.0 b
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.7A. Black rot incidence and severity in pineapple fruit inoculated with a yeast mixture containing *Pichia guilliermondii* (Pichia), *Rhodoturula sp.*(Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus) and *Chalara paradoxa* (CP) as affected by low temperature (10°C) storage for one week.[a] Fruit were held at room temperature (22°C) for 7 days then evaluated.

Treatment	Incidence [b]	Severity [c]
Sterile distilled H_2O	0 b	0 d
Sterile distilled H_2O + CP, 2 hr later	100 a	35 a
Ymixt + CP, 2 hr later, RT 1 wk	100 a	21 b
Ymixt + CP, 2 hr later, 10°C 1 wk	100 a	0 d
Ymixt + CP, 2 hr later 10°C 1 wk , RT 1 wk	100 a	8.5 c
Analysis of Variance Pr > F	0.0001	0.0001

[a] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

Table 5.8A. Black rot incidence and severity in pineapple fruit inoculated with an isolate of *Pichia guilliermondii* (Pichia) or a yeast antagonist mixture containing *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus) and *Chalara paradoxa* (CP) when combined with a low dose of Bayleton.[a] Fruit were held at 22°C for 7 days then evaluated.

Treatment	Incidence [bd]	Severity [cd]
Sterile distilled H_2O	0 b	0 d
Sterile distilled H_2O + CP, 2 hr later	100 a	22.0 a
Pichia + ½ Bayleton + CP, 2 hr later	70 ab	2.8 bc
Ymixt + ½ Bayleton + CP, 2 hr later	80 ab	5.7 b
½ Bayleton + CP, 2 hr later	90 ab	5.9 b
Full Bayleton + CP, 2 hr later	60 ab	1.4 c
Analysis of Variance Pr > F	0.0001	0.0001

[a] Bayleton was applied as a spray. The full dosage of Bayleton was 0.67 g l^{-1}.

[b] Incidence evaluation: percentage of the total number of fruit infected.

[c] Severity evaluation: percentage of surface area that was diseased.

[d] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

Table 6.2A. Radial growth of *Chalara paradoxa* (CP) on glucose yeast extract agar plates with and without yeast antagonists.[a] Plates were held at 22°C for 7 days then evaluated.

Treatment[b]	CP growth diameter (mm)[c]
Sterile distilled H_2O + CP	90.0 a
Pichia + CP	7.0 d
Rhodoturula1 + CP	8.5 d
Rhodoturula2 + CP	18.0 c
Rhodoturula3 + CP	20.0 c
Cryptococcus + CP	31.5 b
Ymixt + CP	7.5 d
Analysis of Variance Pr > F	0.0001

[a] A 100 µl aliquot of yeast suspension (10^8 spores ml^{-1}) or yeast mixture (10^8 spores ml^{-1} of each of five yeast isolates) was spread on the glucose yeast extract agar plate and a 20 µl aliquot of *C. paradoxa* spore suspension (10^6 spores ml^{-1}) was added to the middle of the plate.

[b] The yeast isolates tested were *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus). The yeast mixture contained all five yeast isolates.

[c] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

Table 6.3A. Hyphal growth of *Chalara paradoxa* (CP) on sterile pineapple juice filtrate in plates with and without yeast antagonists.[a] Plates were held at 22°C for 7 days then evaluated.

Treatment [b]	CP growth weight (gm)[c]	
	disc	aliquot
Sterile distilled H_2O + CP	0.35 bc	0.15 b
Pichia + CP	0.14 e	0.02 c
Rhodoturula1 + CP	0.34 c	0.19 ab
Rhodoturula2 + CP	0.37 bc	0.20 ab
Rhodoturula3 + CP	0.43 a	0.23 a
Cryptococcus + CP	0.40 ab	0.23 a
Ymixt + CP	0.21 d	0.07 c
Analysis of Variance Pr > F	0.0001	0.0001

[a] Two 4- mm discs of *C. paradoxa* taken from a 7 day old culture grown at 27°C or a 200 µl aliquot of *C. paradoxa* spore suspension (10^6 spores ml^{-1}) was added to 10 ml sterile pineapple juice filtrate in plates amended with a 100 µl aliquot of yeast suspension (10^8 spores ml^{-1}) or yeast mixture (10^8 spores ml^{-1} of each of five yeast isolates) .

[b] The yeast isolates tested were *Pichia guilliermondii* (Pichia), *Rhodoturula sp.* (Rhodoturula1, Rhodoturula2, Rhodoturula3), and *Cryptococcus sp.* (Cryptococcus). The yeast mixture contained all five yeast isolates.

[c] Data were analyzed using Waller-Duncan K-ratio T test. Means within a column followed by the same letter were not significantly different (n = 10).

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